



# **AN INVESTIGATION OF THE MOLECULAR STRUCTURE, COMPOSITION AND BIOPHYSICAL PROPERTIES OF GUM ARABIC**

**IBRAHIM BABALE *GASHUA*** (BSc, MTECH.)

A thesis submitted in partial fulfilment of the  
requirements of the University of Wolverhampton  
for the degree of Doctor of Philosophy

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## ABSTRACT

*Acacia senegal* and *Acacia seyal* are important agroforestry cash crops indigenous to several countries of sub-Saharan Africa, including Nigeria. The gum exudate produced by these species is termed gum Arabic which is an approved food additive (E414), primarily used as an emulsifier.

In the current study, the molecular structure, composition and biophysical properties of gum samples harvested from mature trees of *Acacia senegal* at two specific ecolocations in Nigeria (NG<sub>1</sub> and NG<sub>2</sub>), have been investigated together with two previously characterised gum samples harvested from *A. senegal* and *A. seyal* originating from Sudan.

The monosaccharide sugar composition analyses have shown that the *A. seyal* gum had a lower rhamnose and glucuronic acid content than the *A. senegal* gum, but had higher arabinose content. No significant difference was observed between the sugar composition of the *A. senegal* gums from Sudan and Nigeria. The total protein content of the Nigerian gum samples were significantly higher than recorded for the Sudanese samples. The principal amino acids present in all the gum samples are hydroxyproline, serine, aspartame, threonine and proline which is in agreement with literature values.

The hydrodynamic size of the molecules present in the gums was studied using dynamic light scattering and it was found that

molecular association occurred in solution over time which was inhibited in the presence of an electrolyte. The comparison of droplet size distribution for emulsions prepared with *A. senegal* (NG<sub>1</sub>) and *A. seyal* gum samples showed that *A. senegal* sample was a better emulsifier than the *A. seyal*.

Multilayer adsorption of the samples onto polystyrene latex particles was observed, which resulted in an increase in thickness of the adsorbed layer as a consequence of the interaction between the protein and carbohydrate within the molecules adsorbed on the emulsion surface.

Preliminary analyses of the gums using transmission electron microscopy showed the presence of varied macromolecules, ranging in size from ~12 - ~60 nm. Immuno-gold negative staining (using JIM8 monoclonal antibody) indicated clear labelling of arabinogalactan-proteins present in the gums harvested from *A. senegal*, the labelling of the *A. seyal* sample was inconclusive.

In summary, the data presented represents the first detailed comparison of the structure, composition and physicochemical characteristics of Nigerian *Acacia* gum exudates versus Sudanese samples (main global supplier) which have shown that gum obtained from Nigerian sources is a viable alternative to ensure future supply of this valuable natural resource.

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This research work is dedicated to:

- The memory of my loving parents, Alhaji Babale Gashua and Adama Babale, who encouraged and instilled in me the value of education.
- All members of the Babale Family, particularly those who will be encouraged by my educational attainments and God bless them.

## LIST OF PEER REVIEWED PUBLICATIONS

1. I.B. Gashua, P.A. Williams, M.P. Yadav and T.C. Baldwin (2015). Characterisation and molecular association of Nigerian and Sudanese Acacia gum exudates. *Food Hydrocolloids*, **51**, 405–413.
2. I.B. Gashua, P.A. Williams and T.C. Baldwin (2016). Molecular characterisation, association and interfacial properties of gum Arabic harvested from both *Acacia senegal* and *Acacia seyal* (*Food Hydrocolloids*, In-Press).

## **LIST OF CONFERENCES ATTENDED**

1. Fractionation of gum Arabic from various sources. 17<sup>th</sup> Gums and Stabilizers for the Food Industry Conference. Glyndwr University, Wrexham, United Kingdom. 25<sup>th</sup> - 29<sup>th</sup> June, 2013.
2. Physicochemical characterisation of gum Arabic and its proteinaceous fractions. 12<sup>th</sup> International Hydrocolloid Conference. National Taiwan University, Taipei, Taiwan (R.O.C). 5<sup>th</sup> - 9<sup>th</sup> May, 2014.
3. Association behaviour of Nigerian and Sudanese *Acacia* gum exudates. 18<sup>th</sup> Gums and Stabilizers for the Food Industry Conference. Glyndwr University, Wrexham, United Kingdom. 23<sup>rd</sup> - 26<sup>th</sup> June, 2015.

## ABBREVIATIONS

µm	micro metre
µL	micro Litre
°C	Degree Celsius
AFM	Atomic Force Microscopy
AG	Arabinogalactan
AGP	Arabinogalactan-protein
BSA	Bovine serum albumin
FPC	Flaxseed protein concentrate
g	grams
GP	Glycoprotein
GPC	Gel Permeation Chromatography
HPAEC-PAD	High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection
HPSEC	High performance size exclusion chromatography
HRGPs	Hydroxyproline-rich glycoproteins
HSM	High shear mixing
KDa	Kilo Daltons
LS	Light scattering
ln	Natural logarithm
MALLS	Multi-Angle Laser Light Scattering
mL	millilitres
Mn	number-average molecular weight
Mw	weight average molecular weight

NCF	Nitrogen conversion factor
nm	nanometres
O/W	oil-in-water
O/W/O	oil-in-water-in-oil
RRIN	Rubber Research Institute of Nigeria
RI	Refractive Index
R.M.S	Root Mean Square
R <sub>g</sub>	Radius of gyration
R <sub>h</sub>	Hydrodynamic radius
SAXS	Small angle X-ray scattering
SDS	Sodium dodecyl sulphate
SDW	Sterile deionised water
SPC	Soybean protein concentrate
STEM	Scanning transmission electron microscopy
SSA	Specific surface area
TBS	Tris buffered saline
TEM	Transmission electron microscopy
TFA	Trifluoro acetic acid
UV	Ultra Violet
W/O	water-in-Oil



## **1.0. Chapter One**

### **1.1.0. General Introduction**

#### **1.1.1. Introduction to plant gum exudates and gum Arabic**

Plant gum exudates are defined as water soluble, complex acidic polysaccharides that are extracted from marine and land plants (either spontaneously or after mechanical injury), that possess the ability to contribute viscosity or gelling ability to dispersions (Abu Baker, Tahir and El-Kheir, 2007). Various plant species can yield the complex polysaccharides commercially known as 'plant based gums'. Studies on several of these (mainly plant gum exudates and seed gums) has given rise to the identification of valuable natural sources of complex carbohydrate polymers with attributes which make them of use for industrial applications (Mirhossein and Amid, 2013).

Within the food industry, the following plant gum exudates are of major commercial importance, gum Arabic (Source: *Acacia senegal* and *Acacia seyal*), Tragacanth gum (Source: *Astragalus gummifer*), gum Ghatti (Source: *Anogeissus latifolia*), gum Karaya (Source: *Sterculia urens*), Grewia gum (Sources: *Grewia mollis*) and Mesquite gum (Source: *Prosopis spp.*) of which, gum Arabic currently holds the greatest commercial importance and as such has been the most extensively studied (FAO, 2015).

Gum Arabic is harvested from *Acacia* trees (see Figure 1.1); and was defined by the Joint Expert Committee on Food Additives of

the Food and Agricultural organisation (JECFA/FAO) in 1999 as 'the dried exudate obtained from the stems and branches of *Acacia senegal* (L) Wildenow or *Acacia seyal* (Family *Leguminosae*)' (Mhinzi, Mghweno and Buchweishaija, 2008).

Gum harvested from *A. senegal* is referred to as Hashab, while that harvested from *A. seyal* is known as Talha. They are both recognised by the Codex Alimentarius and have been assigned the food additive code E414 (UNCTAD, 2009; Abdel-Gadir, et al., 2014).



**Figure 1.1.** Nodules of gum Arabic formed on a wounded branch of *A. senegal* (Source: Cecil, 2005).

Gum Arabic is considered the oldest and best known of all natural gums because it has been used as an article of commerce since the time of the ancient Egyptians over 5,000 years ago (Verbeken, Dierckx and Dewettick, 2003).

The ancient Egyptians referred to the exudate as *kymt*, and its usage was fundamental to numerous processes performed during that time period (Baldwin, 2002). In particular, it was used in the mummification processes as its composition facilitated the preservation of the bodies, disguised the odour of decomposition and aided the temporary preservation of soft tissues (Bretell et al., 2015). Alternative uses for gum Arabic during this period included; in ink for hieroglyphics, in which it was used as a sizing agent (sizing agents are used to reduce the penetration of liquids in to paper) (Globest, 2014). The Greeks and Romans who visited and traded with Egypt also utilised gum Arabic. During Roman mortuary procedures, the gum was used in a similar way to the mummification processes used by the Egyptians. The Greeks modified the Egyptian word *kymt* to *kommi*. From then onwards, the terminology used to refer to the gum continued to change, the Romans referred to it as *gummi*, which the French later changed to *gomme*. Eventually the term 'gum' came to define not only *Acacia* exudates, but all of the 'gummy' substances used in commerce. Thus, gum Arabic - in which Arabic is a reference to the ports used for the shipment of the gum (ISC Gums, 2014).

Due to its ability to stabilise emulsions, act as a thickening agent and its excellent emulsifying properties; gum Arabic's use today extends into various industries (Ali et al., 2012). The pharmaceutical, cosmetic, food, lithography, pottery and textile

industries all utilise gum Arabic in the production of a wide variety of commercial goods (Ali, Zaida and Blunden, 2009). However, it is in the food industry that the majority of gum Arabic is utilised.

### **1.1.2. The genus *Acacia***

*Acacia* is one of the largest vascular plant genera and encompasses a wide range of ecological environments (Abdel-Farida, Sheded and Mohamed, 2014) species of which can be found in Australia, Africa, India and America (Brown, Warwick and Prychid, 2013). The environmental conditions experienced in these countries range from arid deserts to tropical climates (Brown, Warwick and Prychid, 2013). Botanically speaking, the genus is a part of the *Mimosoideae* subfamily, which is a large pantropical sub-family (Abdel-Farida, Sheded and Mohamed, 2014). Its name is derived from the Greek word for thorns '*akakia*' (Borzelleca and Lane, 2008; Chiveu et al., 2008).

There are more than a thousand species of *Acacia*, which are classified into three major groups; subgenus *Acacia (gummiferae)* containing ~120 - 30 species; subgenus *Aculeiferum (Vulgares)* with ~180 - 190 species and subgenus *Phyllodinae* with more than 900 species (Ali et al., 2012). According to Coppen, (1995), *Acacia senegal* is generally regarded as having four different varieties namely *A. senegal* (L.) Willd. var. *senegal* (syn. *A. verec* Guill.); *A. senegal* (L.) Willd. var. *kerensis* Schweinf; *A. senegal* (L.) Willd.

var. *rostata* Brenan; and *A. senegal* (L.) Willd. var. *leiorchis* Brenan (syn. *A. circummarginata* Chiov.); while *Acacia seyal* is comprised of only two varieties: *A. seyal* Del. var. *seyal* and *A. seyal* (Del.) var. *fistula* (Schweinf) Oliv.

Species within the genus are classified as deciduous shrubs (shrub trees), which grow to approximately 2 - 5 m in height, with a flat or round crown. The bark of these trees is typically yellow-brown in colour and is smooth in texture on younger trees, changing to dark green, gnarled and cracked on mature specimens. Although the species within the genus can be found in a wide range of climatic zones, the majority are found in arid locations. Phenotypically, *Acacia* trees are well adapted for survival in the harsh environments in which the majority of species are found (Lorenzo, Gonzalez and Reigosa, 2010). Their extensive root systems enable them to thrive in soils in which the nutrient content is poor. They exhibit soil-stored (long term) seed banks, generalist seed-dispersal syndromes and generalist pollination syndromes (Lorenzo, Gonzalez and Reigosa, 2010).

All the gum yielding species within the genus are phenotypically quite similar, and inhabit climatic regions that are largely uniform. These include: *Acacia nilotica*, *A. seyal*, *A. senegal*, *A. eampylacantha*, *A. karoo*, *A. glaucophylla*, *A. abyssinica*, *A. gummifera*, *A. arabica* (Grieve, 2014). As mentioned previously, the most important gum yielding species in terms of their global

agronomic importance are *A. senegal* and *A. seyal*, with *A. senegal* widely recognised as the more important of the two.

### **1.1.3. *Acacia senegal***

This species is native to the semi desert regions of sub-Saharan Africa, and is mostly found in the Sudano-Sahelian zone of Africa from Sudan to Senegal (Khalafalla and Daffalla, 2008). This is a very variable taxon, presenting difficult taxonomic uncertainties and unresolved contradictions (Mulumba and Kakudidi, 2011; Kyalangalilwa et al., 2013) in its classification. Varietal differences within the species are based upon variation in natural distribution as well as differences in morphological characteristics such as the presence or absence of hair on the axis of the flower, colour of the floral axis, shape of seed pod tips, occurrence of a distinct trunk and shape of crown. *A. senegal* often has compound leaves consisting of numerous small leaflets and bears clusters of yellow or white flowers with flattened pods, flowering begins before or at the beginning of the rainy season when the leaves emerge and the seeds mature in the dry season (see Figure 1.2).



**Figure 1.2.** Pod, Seeds, Leaves and Flowers of *Acacia senegal* (Dauqan and Abdullah, 2013).

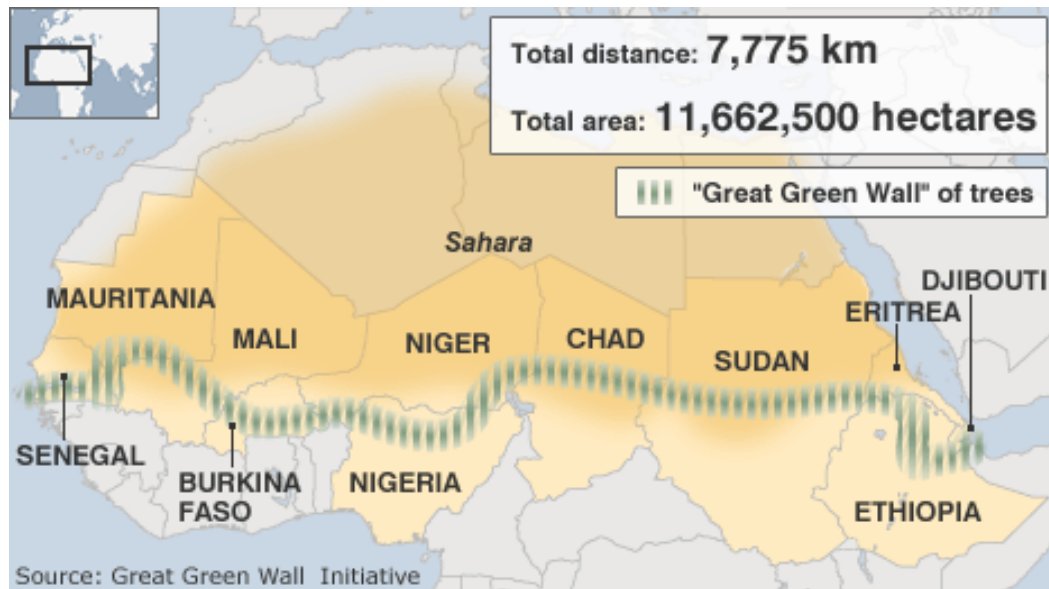
In areas with more than one variety, there can be large variations in flowering and fruiting time; pollination is probably by insects (Kyalangalilwa et al., 2013). Its mode of propagation under natural conditions is by seeds, the winds shakes seeds from the dehiscent pods, and sheet wash and grazing animals may extend the seed dispersal range, although distribution of the plant is limited by poor germination and premature death of young seedlings (Khalafalla and Daffalla, 2008).

This species has acquired a wide geographical distribution due to its drought tolerance. Moreover, as a legume, its' ability to fix atmospheric nitrogen also allows it to grow on nitrogen poor soils. In its countries of origin, *Acacia senegal* is also known for its ability to prevent desertification, by stabilizing sand dunes and serving as a wind break (Chiveu et al., 2008).

As mentioned previously, gum Arabic is a plant gum exudate, harvested primarily from wounded stems and branches of *Acacia senegal* and *Acacia seyal*, both of which are indigenous to sub-Saharan (Sahelian) Africa (Rahim, Rubem and Ierland, 2005; Valmar, 2008). Both species are found growing across the entire length of the Sahel, forming the 3000 km long and 15 km wide 'gum Arabic/green belt' of Africa (see Figure 1.3 below).

*Acacia senegal* which is viewed as the 'best' source of gum Arabic, is extremely resistant to water stress and is tolerant of rainfall and temperature variations that commonly occur in the Sahelian regions of Africa. As such, it is able to grow in areas with an annual rainfall between 100 - 950 mm (average 300 - 400 mm) and 5 - 11 months dry season. It can tolerate daily temperatures of up to 50 °C, dry winds and sand storms (Valmar, 2008). The plant prefers coarse textured soils, but it will also grow on slightly loamy sands. In addition to the Sahelian region of Africa, *A. senegal* also grows in the Sind and Ajmere regions of India, both of which produce very small quantities of gum Arabic, mainly for local use.





**Figure 1.3.** 'Great green wall' of trees in Africa showing the countries where gum Arabic trees (*Acacia senegal* and *Acacia seyal*) are found both as commercial plantations and in the wild (Adapted from: Green Planet, 2015).

*A. senegal* is considered to be an important agro forestry cash crop in several countries including Sudan, Nigeria and Chad. It is also used as a fuel wood, whilst the seed pods and foliage serve as a source of animal feedstuff.

It has been reported (Hussein, 1983; Badi, Ahmed and Bayomi, 2009) that trees of *Acacia senegal* grown in western Sudan are managed in a time sequence through intercropping with crop species such as millet, sorghum, sesame, groundnut and water melon. This agro forestry system plays an important role in environmental conservation and also makes full use of the

available resources compared to when the tree is grown as a monoculture (Fadl, 2010).

#### **1.1.4. Gummosis**

Gummosis is a common response to wounding, that results in the exudation of a plastic gum sealant from wounds caused as a reaction to external stimuli such as adverse weather conditions, pathogen attack, predation or mechanical damage (Joseleau and Ullmann, 1990).

Ballal et al., (2005b) observed that both the synthesis and exudation of gum Arabic occurs only during dry conditions, (i.e. during the dry season in arid climates). However, the physiological and environmental factors controlling gum yield are still not well understood (Abib et al., 2012; Harmand et al., 2012).

The systematic wounding of a plant to induce gummosis is referred to as "tapping", and influences gum yield through timing and the tapping intensity. Once tapped, the gum slowly collects in the wound within a 3 - 8 week period, depending upon the weather condition, and a yield of up to 7,000 g can be obtained per tree, on an annual basis (Ballal, et al., 2005a).

Gum formation in *A. senegal* and related species occurs within the cambial zone of the branches and stems, and is induced when the plants/trees are subjected to stress conditions such as heat, drought, insect attack or systemic wounding. This process was

investigated by a comparison of polysaccharides extracted from the inner bark, cambial zone and the xylem of a wounded (tapped) region, to those present in an unwounded (untapped region). The three anatomical zones were successfully extracted for water-soluble polysaccharide and alkali-extractable hemicellulose. The results of which demonstrated the presence of gum in the cambial zone, on the boundary (side) of the scarified region of the tree and also a short distance beyond the limits of the tapping (Joseleau and Ullmann, 1990).

Commercially, trees are tapped when they begin to lose foliage, as this is an indication that the required low water threshold has been reached (Abib, 2012). It is during this period that gum production will be at its greatest. Studies have also suggested that if tapping commences at a later stage in the dry season, then gum production is reduced, thus indicating an 'ideal' time frame during which tapping should occur (Abib, 2012). In addition to favourable climatic conditions; the age of the tree is also thought to directly correlate with gum production. Studies suggest, that only mature trees which are >5 years in age, are able to produce gum of marketable quality (Baldwin, 2002).

### **1.1.5. Economic Botany**

Gum Arabic is of importance in international trade because of its use as an additive in confectionary, beverages, pharmaceuticals and many other products (Anderson, 1993).

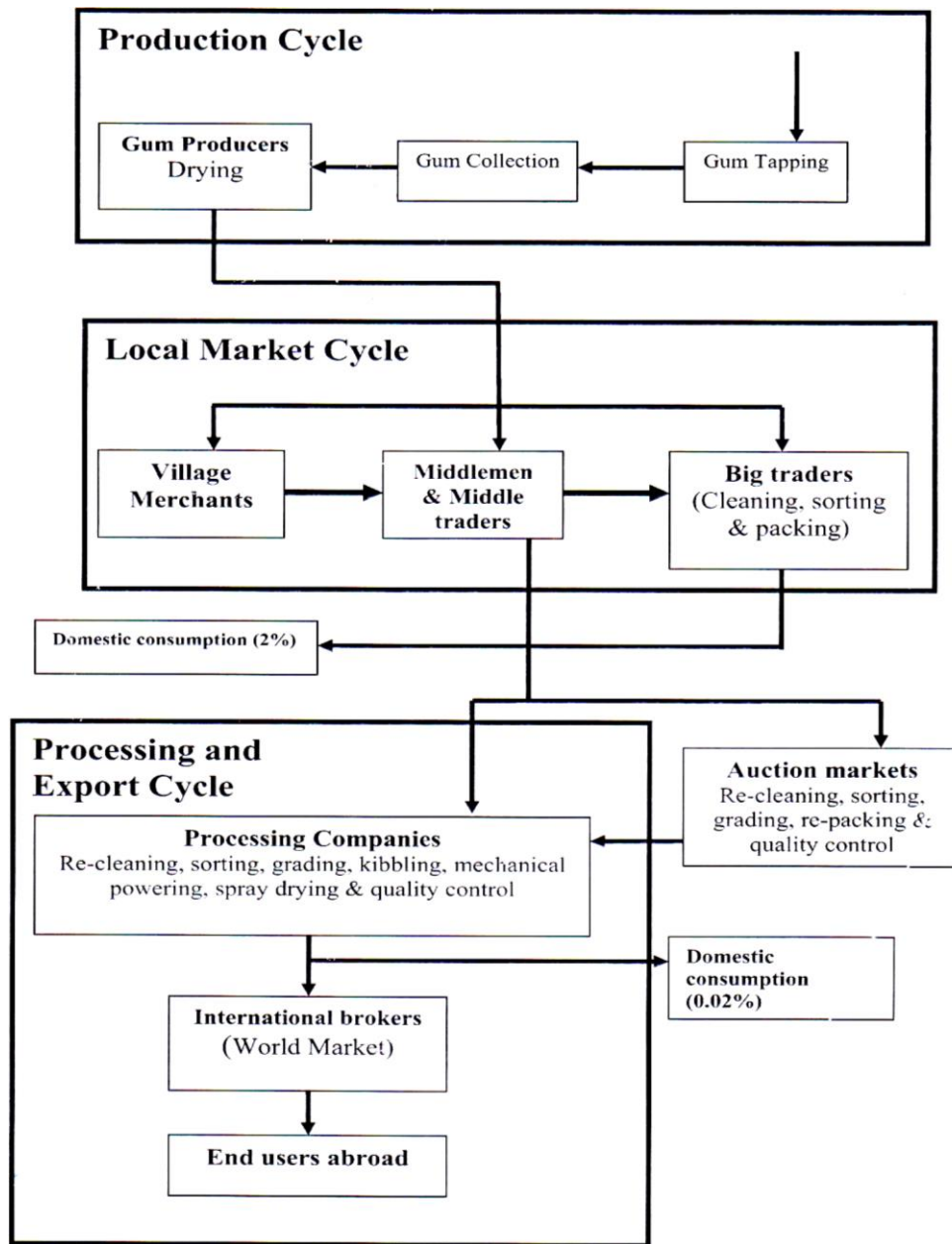
In Sudan, where *Acacia senegal* is found wild, as well as cultivated in agroforestry, the revenue from gum Arabic represents approximately 4% of the total national revenue (Elmqvist et al., 2005). Sudan is considered the worlds' largest producer and exporter of gum Arabic, (contributing 80 - 90% of the global supply). However, due to political and humanitarian crises over the past twenty or so years, gum Arabic production in Sudan has become unreliable, with average production declining from approximately 60,000 metric tonnes in the 1960s to less than 20,000 metric tonnes in the 1990s (Abdul, 2006). However, in recent years coinciding with some improvements in the political situation, production has risen to approximately 55,000 tonnes in 2010 (Martelli, 2010) and it is estimated that exports of this commodity may have exceeded 100,000 tonnes in 2014 (Abdel-Gadir, et al., 2014).

In Nigeria, the annual average production Figures for gum Arabic for 2002 and 2004 were estimated to be 16,071 and 17,206 tonnes respectively (RMRDC, 2004) and rose to approximately 20,000 tonnes in 2005, making Nigeria the second largest producer, after Sudan (Ademoh and Abdullahi, 2009). A tonne of Nigerian raw

gum Arabic was valued at USD 1,500 in 2005, USD 3,000 in 2008 and dropped to USD 1,961 in 2009, USD 1,615 in 2010 and USD 1,356 in 2011. This recent decrease in value is due to the global economic recession and higher production Figures in Sudan (ITC, 2011). A unique feature of gum Arabic export in Nigeria, is that India is its second largest customer, with a marked recent increase in exports to that country, from 300 tonnes in 2006 to 5784 tonnes in 2010 (UNCTAD, 2009; Sagay and Mesike, 2011).

In terms of importation, the USA is the largest importer, accounting for up to 30% of the total gum Arabic trade (ITC, 2011). Gum Arabic is harvested from the source plant manually and thus, often contains extraneous material such as sand, and pieces of bark which must be removed prior to use. According to Islam et al., (1997), the commercial grades of gum exudates obtained from *Acacia* species vary considerably in quality. The best commercial grades of gum are defined as being clean, highly water-soluble and give colourless or pale yellow aqueous solutions. The various processes involved in the production and marketing of gum Arabic are presented in Figure 1.4. Gum produced for export is marketed in various grades. Hand Picked Selected is the highest grade (grade one) and contains the cleanest and largest gum nodules, with the lightest colour and therefore commands the highest price. This top grade of gum is defined as possessing an optical rotation of  $-26$  to  $-34^{\circ}$  and a nitrogen content of 0.27 to

0.39% (Anderson, 1993; Mahendran, et al., 2008). Cleaned and sifted grade (grade two) comprises the material which remains after Hand Picked Selected and the siftings have been removed. Cleaned is the third 'standard' grade used throughout the world, where the colour varies from light to dark amber and the gum contains various amount of siftings, but without dust. Siftings are the residue formed by removing the other choice grades. Dust constitutes the lowest grade, and is collected upon completion of the cleaning process, and comprises very fine particles of gum admixed with sand and dirt (Islam, 1997).



**Figure 1.4.** Gum production and marketing channel in Sudan (Elrayah, et al. 2012). The process starts with tapping the gum from the tree and allow to solidify (after exposure to air) before collecting it from the plant and subsequently drying it under shade by the gum producers.

#### **1.1.6. Chemical composition and molecular studies of gum Arabic**

The physical and functional properties of plant-based gums depend upon their chemical composition and molecular structure (Mirhosseini and Amid, 2012). Recent literature suggests that there is growing interest in the relationship between the chemical composition, molecular structure, physical characteristics and functional properties of plant gum exudates.

Chemically speaking, gum Arabic is a slightly acidic complex compound, comprised of polysaccharides, glycoproteins and their calcium, magnesium and potassium salts (Flindt et al., 2005). The chemical composition, which also determines the quality of the gum vary depending on its geographical origin, weather conditions at the time of harvest, soil, age and genotype of tree and the processing conditions (Idris et al., 1998; Flindt et al., 2005; Josiah et al., 2008; Yebeyen, Lemenih and Faleke, 2009; Lelon et al., 2010; Habeballa, Hamza and El Gaali, 2010; Harmand, 2012).

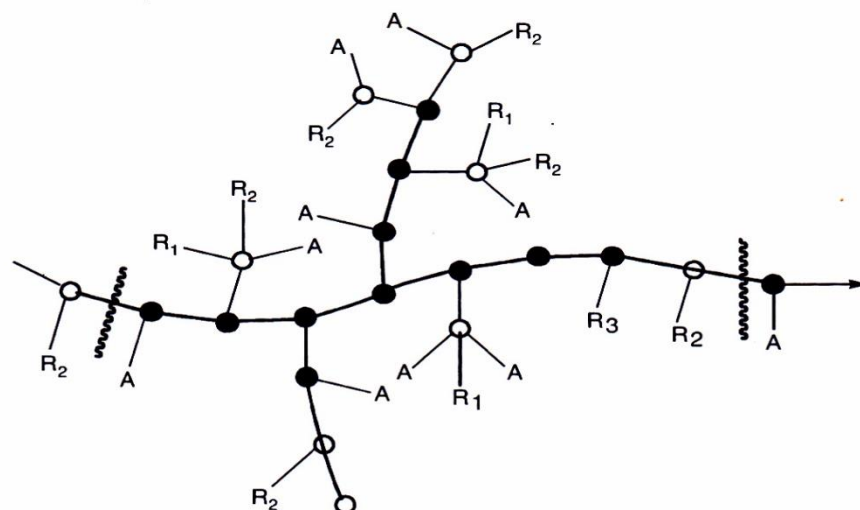
Previous studies of the molecular structure and composition of gum Arabic (i.e. gums harvested from both *Acacia senegal* and *Acacia seyal*) which will be discussed in more detail later, have revealed that the gum consists of three major components which include an arabinogalactan peptide fraction (AG), with a molecular weight of approximately 300 kDa and a very low protein content (Renard, et al., 2006; Sanchez, et al., 2008), arabinogalactan protein (AGP)



fraction with a molecular weight of  $\sim 1400$  kDa (Goodrum, 2000), and a small proportion of glycoprotein(s) (GP) whose amino acid composition differs from that of the AGP component (Williams, Phillips and Stephen, 1990).

Although the carbohydrate content of the three major components is similar, the protein-rich fractions have a lower glucuronic acid content and only the AGP and GP components have a secondary structure (Williams Phillips and Stephen, 1990; Renard, et al., 2006). The estimated amount of the monosaccharide constituents are arabinose (27%), rhamnose (13%), galactose (44%) and glucuronic acid (16%) (Renard et al., 2014).

The complex chemical and macromolecular structure/composition of the gum has been widely studied, for many years, and has been shown to consist mainly of a highly branched polysaccharide complex with protein as a minor component. The polysaccharide back bone of the gum is composed of 1,3-linked  $\beta$ -D-galactopyranosyl units. The side chains are composed of two to five 1,3-linked  $\beta$ -D-galactopyranosyl units, joined to the main chain by 1,6-linkages. A typical model of the molecular structure of *Acacia* gum is presented in Figure 1.5.



A = Arabinosyl  
 ● = 3-Linked Galp,    ○ = 6-linked galp, or end-group  
 R<sub>1</sub> = Rha → 4GlcA,    R<sub>2</sub> = Gal → 3 Ara, R<sub>3</sub> = Ara → 3 Ara → 3 Ara

(b)

**Figure 1.5.** Schematic representation of the Molecular structure of gum Arabic showing the 3 and 6-linked Galp and the associated monosaccharides. The mark at the end of the structure indicates the point of attachment of another molecule. (Adapted from Cui, et al., 2005).

Akiyama, Eda and Kato, (1984) were the first to demonstrate that both hydroxyproline and serine residues are present in the gum and that a proportion was covalently attached to carbohydrate moieties. They also found that the gum precipitated with  $\beta$ -glucosyl Yariv reagent, confirming that it is a type of arabinogalactan protein complex.

Isolated hydroxyproline residues appear to be the point of attachment for polysaccharide chains, whereas clusters of

hydroxyproline are sites attachment for oligoarabinose chains containing four to six residues (Mahendran et al., 2008).

However, despite the deluge of papers that have been published over the past fifty years on the chemical structure and composition of gum Arabic e.g. Karamalla, Siddig and Osman, (1965); Aspinall, (1970); Churms, Merrifield and Stephen, (1983); Conolly, Fenyo and Vandeveld, (1988); Menzies, (1992); Islam et al., (1997); Williams and Phillips, (2000); Cui, et al., (2005); Mahendran, et al., (2008); Sanchez, et al., (2008); Ali, Zaida and Blunden, (2009); Renard, et al., (2010); Funami, (2010); Renard, et al., (2012), etc the detail of which will be described in later chapters; the exact structure is yet to be completely resolved (Klein et al., 2010; Rodge, et al., 2012; Nie, et al., 2013)!

#### **1.1.7. Serotaxonomic studies**

Whilst most studies of gum Arabic and related *Acacia* gum exudates (and seed proteins) have focused purely on the chemical, physicochemical and structural properties of these substances, a number of chemo/Serotaxonomic investigations have also been reported. Anderson (1978) was the first to suggest that the biochemical and biophysical data on *Acacia* gum exudates could be used to augment the classical botanical classification of *Acacia* based solely on external morphological features such as the 'classic' monograph of Bentham (1875). The first report to follow

up on Anderson's suggestions was published by El Tinay et al., (1979). In the study, seed proteins harvested from 22 species of *Acacia* collected from Northern, Central and Western Sudan were compared by serological methods in an attempt to classify Sudanese *Acacia* species. From the results of the study using polyclonal antisera raised against each sample, the seed proteins were divided in to two main groups and six sub-groups.

Subsequently, Brain reported a study using immunological techniques to investigate *Acacia* phylogeny, whereby the seed proteins of 37 species of *Acacia* were tested serologically by double diffusion and immuno electrophoresis using rabbit antisera raised against whole seed contents of *A. karoo*, *A. ataxacantha* and *A. mearnsii* (Brain, 1987). Identity and absorption tests showed remarkable homogeneity in the *Gummiferae* series. In the study, the seed proteins of *Acacias* from Africa and Australia were analysed, and were found to have virtually identical reactions with the antisera. In terms of the evolution and diversification of the *Gummiferae Acacia*, it was remarkable that there was so little difference in the seed proteins of these species despite geographical separation for millions of years (Brain, 1987).

The first detailed description of the use of anti-gum Arabic antibodies in an immunoassay was described by Pazur, et al., (1986). Since which time, several similar reports have been published (Pazur et al., 1991; Miskiel and Pazur, 1991; Williams et

al., 1992; Osman et al., 1993a). In addition, a sensitive and specific ELISA for *A. senegal* gum has been reported which could differentiate this gum from other commonly known food hydrocolloids including other plant gum exudates such as Ghatti, Tragacant and Karaya (Williams et al., 1992; Menzies et al., 1992). Further studies of *Acacia* gum using this ELISA in combination with a range of chemical and physicochemical techniques indicated that the interaction with anti-gum Arabic antisera could be correlated with differences in molecular composition of the gums. These studies suggested the utility of such immunoassays for chemotaxonomic studies of *Acacia* (Osman et al., 1993a). The only major drawback was that only a finite quantity of the polyclonal antisera was available. However, in the same year, Osman and his colleagues also reported the use of a panel of anti-arabinogalactan/arabinogalactan protein (AG/AGP) monoclonal antibodies in conjunction with a range of chemical/physicochemical analyses to study the molecular composition of gum Arabic (Osman et al., 1993b). This and later studies (Osman et al., 1995; Menzies et al., 1996; Baldwin, Quah and Menzies, 1999) clearly demonstrate the utility of anti-plant cell wall monoclonal antibodies in analyses of the macromolecular composition of gum Arabic and fractions derived from it, and also the ability of such antibodies to distinguish between gum exudates harvested from wide variety of *Acacia* species.

### **1.1.8. Emulsification properties**

The main role of an emulsifier is to adsorb at the surface of freshly formed fine droplets, and prevent them from coalescing with their neighbours to form larger droplets. For a fixed rate of energy dissipation during emulsification, the final droplet size distribution is determined by the time taken for the interface to be covered with emulsifiers, as compared with the average time interval between droplet collisions. When emulsifiers adsorb too slowly, or are present at too low concentration, most of the individual droplets formed during the intense energy dissipation of emulsification are not retained in the final emulsion (Dickinson, 2009b).

In view of the aforementioned explanation of emulsification, gum Arabic is extensively used as an emulsifier/stabilizer in beverage emulsions for soft drinks (Tan, 2004). Chemically speaking, the AGP fraction has been shown to be responsible for the emulsifying properties of gum Arabic (Randall, Phillips and Williams, 1989).

The emulsification capacity of gum Arabic is known to be influenced by a number of factors; such as climatic conditions during harvesting, age and genotype of the tree and storage conditions. These issues affect the composition and molecular weight of the gum Arabic and consequently, its quality (Idris, Williams and Phillips, 1998; Flindt et al., 2005).

Gum Arabic displays a unique combination of excellent emulsifying properties and a low solution viscosity, which makes it very useful in several industries; the food industry in particular, where it is widely used as a flavour encapsulator and stabilizer of oil emulsion concentrates in soft drinks (Yadav et al., 2007). Degen, Vidoni and Rehage, (2012), observed that, the presence of both hydrophilic sugar residues and hydrophobic amino acids are the main components which give it the ability to adsorb at oil-water interfaces.

In emulsions, the hydrophobic polypeptide chains are adsorbed at the oil - water interface, while the hydrophilic polysaccharide protrudes into the aqueous phase to form the dense layer (Buffo, Reineccius and Oehlert, 2001). Erni et al., (2007) earlier studied the elastic behaviour of the adsorption layer of gum Arabic and suggested that the elastic behaviour plays a significant role in the stabilisation of oil/water emulsions. Gum Arabic is also able to form thick viscoelastic films, by adsorbing onto the oil-water interface, thereby reducing the interfacial tension between oil and water thus performing as an emulsifying agent (Tan, 2004).

#### **1.1.9. Molecular association/aggregation**

Some polysaccharides have the tendency to associate in aqueous solution and this affects their functionality and industrial applications, due to its influence on the molecular weight and size

of the molecule, which also determines how the molecules interact with each other.

Montenegro, et al., (2012), reported that interactions such as hydrogen bonding, hydrophobic association and electrostatic interactions, depend upon the concentration and presence of protein components that affect the ability to form supramolecular complexes, all of which are properties possessed by gum Arabic.

Studies by Al-Assaf, et al., (2007), showed that, molecular association in gum Arabic can lead to an increase in molecular weight in the solid state by maturation under controlled conditions of heat and humidity. In another study by Al-Assaf and his colleagues, the role of protein components present in the gum in enhancing molecular association was investigated under different processing conditions. It was observed that these protein moieties promote molecular association through hydrophobic interactions that influence the size and proportionality of the high molecular weight AGP, thus improving its emulsifying potential (Al-Assaf et al, 2009).

#### **1.1.10. Uses and Applications**

As mentioned earlier, plant gum exudates such as gum Arabic are extensively used in a variety of industrial applications, due to their emulsification, microencapsulation, thickening and stabilization



properties. Gum Arabic finds wide application as a flavour encapsulator in dry mix products such as puddings, desserts, cake and soup mixes and is also used to emulsify essential oils in soft drinks and too prevent sugar crystallization in confectionary products (CNI, 2005).

Among the advantages of natural gums over their synthetic counterparts are their biocompatibility, low cost, low toxicity and relatively wide spread availability (Nep and Conway, 2010).

Gum Arabic is extensively utilized in the food industry where it is used as food additive to impart desirable properties through its influence on the viscosity, body and texture of food. In addition, it is nontoxic, odourless, colourless, tasteless and completely soluble in water and does not affect the flavour, odour, colour of the food to which it is added (Tewari, 2010). The absence of toxicity gives it unique characteristics among other food hydrocolloids.

In food that contains fat and/or oil, gum Arabic is used as emulsifier to maintain uniform distribution of the fat through the product. While as described previously, in the manufacture of soft drinks, it is suitable for use in flavour oil emulsions, where it prevents flocculation and coalescence and inhibits destabilization caused by creaming (Tadesse, Desalegn and Alia, 2007; Wyasu and Okereke, 2012).

Microencapsulation is the process whereby particles of an active ingredient are formed and covered with a thin layer of another material, thus providing protection and controlled release. This has been one of the most important uses of gum Arabic in the food and pharmaceutical industries. Gum Arabic is an ideal material in flavour encapsulation due to its emulsifying and surface-active properties. It is used as a fixative in spray drying applications to protect the flavour compound against oxidation and volatilization (Krishnan, Bhosale and Singha, 2005).

Gum Arabic is also used in processed foodstuffs as a source of dietary fibre, as it has shown to conform to the classification of dietary fibre as formally agreed by the European Union and Codex Alimentarius, (Phillips, Ogasawara and Ushida, 2008; Phillips and Philips, 2011), to be made up of fragments of edible plant cells, polysaccharides, lignin and allied substances resistant to (hydrolysis) digestion by the alimentary enzymes in humans.

In summary, the food applications of gum Arabic include use in emulsification, stabilization, encapsulation, water binding, adhesion, film coating and as a source of dietary fibre.

#### **1.1.11. Aims and Objectives**

Over the past century gum Arabic has served as one of the most important commodities of international trade in the food,

pharmaceutical, adhesive, paper, textile and many other industries. Despite the fact that its chemical structure and composition has been so intensively studied over the past fifty years, many aspects, especially in relation to the various protein components present have yet to be fully resolved (Klein et al. 2010). This is of particular significance as it is currently hypothesised that it is the arabinogalactan proteins (AGPs) and glycoproteins (GPs) present in the gum that are of most importance in relation to its use as an emulsifier for flavour oils for application in the food industry. It has been proposed that the emulsification properties of the gum are due to the presence of proteinaceous components which adsorb and anchor the gum molecules onto the surface of the oil droplets; while the carbohydrate components extend into the aqueous phase and prevent coalescence through electro steric repulsive forces (Randall, Philips and Williams, 1989).

Recent studies of the adsorption of gum Arabic onto limonene oil droplets found that the amount adsorbed was  $\sim 5 \text{ mg m}^{-2}$  which is greater than might be expected from monolayer coverage, suggesting that multi-layers may be formed (Padala, Williams and Phillips, 2009).

Multilayer adsorption is unusual and may be the reason why gum Arabic is such an effective emulsifying agent. This may arise from the fact that the gum contains protein as an integral part of its

structure and that multilayers are formed by interaction of the protein-carbohydrate moieties at the interface. These interactions may also be responsible for the increase in viscosity noted for gum Arabic solutions on standing.

In addition, the majority of work on gum Arabic has been focused on gum harvested in Sudan with little work on gum sourced from Nigeria (world's second largest producer). In light of which the aims of the current study were as follows:

1. To characterise two gum Arabic samples (harvested from *A. senegal*) obtained from known eco-locations in Nigeria and to compare their molecular structure, composition and biophysical properties with a well characterised Sudanese gum Arabic samples harvested from *A. senegal* and *A. seyal* from Sudan using gel permeation chromatography (GPC) in conjunction with amino acid and monosaccharide composition analyses.
2. To investigate the role played by the molecular structure and composition of gum Arabic in the mechanism of adsorption at the oil-water interface by determining the thickness of the adsorbed polymer layers via dynamic light scattering.

3. To assess the stability of the emulsions by monitoring the droplet size as a function of time using laser diffraction.
4. To use transmission electron microscopy to investigate the nature and shape of the macromolecules present in gum Arabic.

## **2.0. Chapter Two**

### **2.1.0. Characterisation and physiochemical properties of Nigerian and Sudanese samples of gum Arabic**

#### **2.1.1. Introduction**

Gum Arabic is a complex, polysaccharide-based plant gum exudate, obtained from trees of selected *Acacia* species (i.e. *Acacia senegal* (L.) Wild. and *Acacia seyal* Del.), which are indigenous to the Sahelian region of the African continent, for which Sudan is the world's leading producer followed by Nigeria and Chad (UNCTAD, 2009).

*Acacia* is a 'cosmopolitan' genus containing over 1350 species and constitutes one of the most populous tree genera in the plant kingdom (Maslin, Miller and Seigler, (2003). *Acacia senegal* (*A. senegal*) and *Acacia seyal* (*A. seyal*) are the main commercially exploited species of the genus. As mentioned in Chapter 1, the gum produced by these species is widely used in the food industry as an emulsifier, for flavour oils present in a wide variety of beverages. It is also extensively utilised in confectionery products, in which it is used to control texture and inhibit sugar crystallisation (Williams, et al., 2006; Williams and Phillips, 2009).

The precise molecular composition of the gums harvested from *A. senegal* and *A. seyal* differ, but the molecular structure recorded for the most abundant molecular constituent of both gums (i.e. arabinogalactan), is the same and consists of a core of 1,3-linked

galactose units with branches linked through the 6 position consisting of galactose and arabinose terminated by rhamnose and glucuronic acid (Street and Anderson, 1983; Siddig, et al., 2005; Al-Assaf, Phillips and Williams, 2005a). Both gums also contain a small percentage of proteinaceous material, with an almost identical amino acid composition, some of which is covalently attached to arabinogalactan (Siddig, et al., 2005; Mahendran, et al., 2008).

#### **2.1.2. Gel Permeation Chromatography**

Characterisation using Gel Permeation Chromatography (GPC), coupled to light scattering, refractive index (RI) and ultraviolet (UV) detectors has shown that the gum exudates obtained from both *A. senegal* and *A. seyal* consist of three main fractions, referred to as the arabinogalactan (AG), arabinogalactan protein (AGP) and glycoprotein (GP) components, which differ mainly in their molecular size and protein content (Randall, Phillips and Williams, 1989; Siddig, et al., 2005; Osman, et al., 1993a; Renard, et al., 2006; Mahendran, et al., 2008). The AG, AGP and GP fractions account for ~90%, ~10% and ~1% of the total gum respectively. They have molar masses of ~250 kDa, 1-2,000 kDa, and 200 kDa, and contain <1%, ~10% and 25-50% proteinaceous material respectively.

### **2.1.3. Molecular structure and composition**

The amino acid content/composition of the three components differs significantly, with that recorded for the GP fraction showing least similarity to the other fractions. The major amino acids found in the AG fraction in descending order are Hydroxyproline (Hyp), serine (Ser), Glutamic acid (Glu), Proline (Pro) and Glycine (Gly), the major amino acids in the AGP fraction are Hyp, Ser, Threonine (Thr), Pro, Leucine (Leu) and Histidine (His), whereas the most abundant amino acids in the GP component are Aspartic acid (Asp), Phenylalanine (Phe), Serine (Ser), Glu and Gly (Randall, Phillips and Williams, 1989). These differences suggest that the glycoprotein(s) present in the GP fraction are less likely to include hydroxyproline-rich glycoproteins (HRGPs) than those present in the other two fractions. This is of particular interest, as this is the least intensively studied fraction of gum Arabic, and one or more of these proteins may play an important role in some of the food applications for the gum, such as in oil-in-water emulsions used in the beverage industry.

Considerable progress has been made over the past twenty to thirty years regarding the general understanding of the molecular structure and composition of gum Arabic using fractions isolated by hydrophobic affinity chromatography (Randall, Phillips and Williams, 1989; Siddig, et al., 2005; Renard, et al., 2006). It is important to note that the fractions obtained using this separation



technique have been shown to have a broad molecular mass distribution, but each fraction contains a predominance of either the AG, AGP or GP components (Osman, et al., 1993; Renard, et al., 2006).

Most of the structural studies undertaken on gum Arabic have focused on gum obtained from Sudanese *Acacia senegal*, but very little has been reported regarding Nigerian *Acacia* gums samples. From the literature, it is known that the molecular compositions of *Acacia* gums vary with climatic conditions, soil type and most definitely the location of growth of the parent plant (Idris, Williams and Phillips, 1998; Idris and Haddad, 2012).

#### **2.1.4. Molecular association in solution**

The determination of the precise molecular structure and composition of gum Arabic is further complicated by the fact that the macromolecules present in the gum tend to self-associate when in solution, as demonstrated by Li et al., using dynamic light scattering (Li et al., 2009). These authors hypothesised, that this self-association is brought about through hydrogen bonding, and is responsible for the changes in rheological properties of gum Arabic solutions over time, as also reported by Sanchez et al. (Sanchez, et al., 2002). Molecular association can also occur when the gum is subjected to heat as, for example, during maturation and spray drying as reported by Aoki et al., (2007). They followed the

maturation process using GPC and demonstrated that the intensity of the peaks for the AG and GP fractions decreased and AGP increased over time and this was attributed to association of the proteinaceous moieties present. This is likely to occur through hydrophobic bonding.

In line with this information, the aim of the current study, was to investigate the physiochemical characteristics of two samples of gum Arabic harvested from trees of *Acacia senegal* at two different geographical locations in Nigeria, which have not been studied previously and to compare their structure, composition and physicochemical properties with those of previously studied samples of gum Arabic, harvested from trees of *A. senegal* and *A. seyal* originating from Sudan.

## **2.2.0. Materials and Methods**

### **2.2.1. Plant Materials**

*Acacia senegal* gum exudates were obtained from two provenances/ecological locations in the North Eastern (Sahelian) region of Nigeria namely; Gashua (NG<sub>1</sub>) located on latitude 12°53'10"N, longitude 10°52'38"E and altitude 370m which has a sandy, Sahelian soil type, and at Gujba (NG<sub>2</sub>) located on latitude 11°24'39"N, longitude 11°59'38"E and altitude 456m which has a clay-loam soil type. Both locations are known locally for gum Arabic production, which is mostly harvested from indigenous trees. Both of the Nigerian gum Arabic samples were authenticated by the Rubber Research Institute of Nigeria (RRIN) substation in Gashua. Authenticated Sudanese samples of gum Arabic, harvested from trees of *A. senegal* and *A. seyal* were obtained from the Phillips Hydrocolloids Research Centre, Glyndwr University, United Kingdom. All four gum samples were obtained in the form of raw gum nodules and had not been processed in any way prior to the current investigation.

## **2.2.2. Methods**

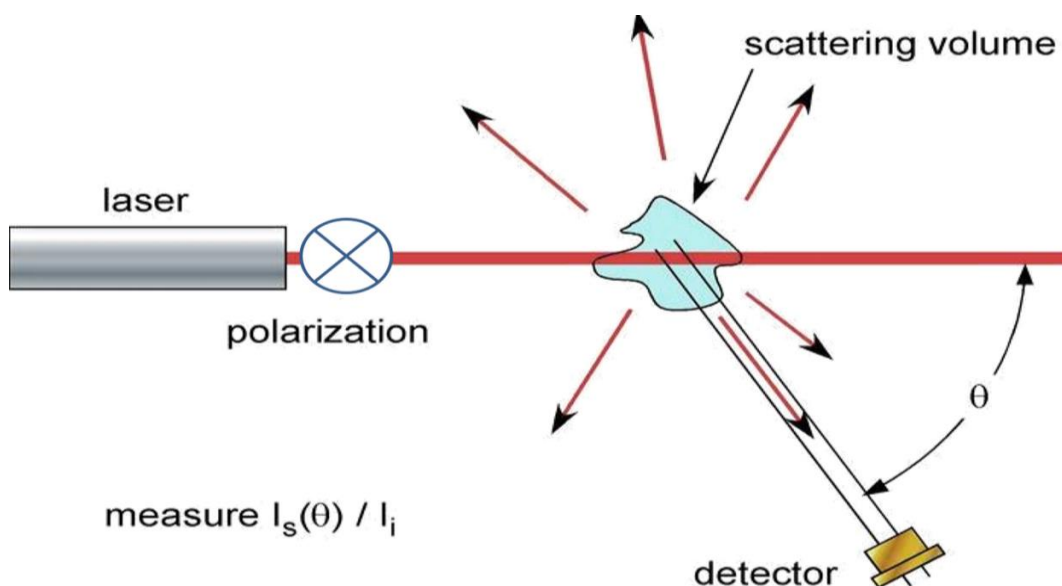
### **2.2.2.1. Gel Permeation Chromatography**

Gel Permeation Chromatography-Multi-Angle Laser Light Scattering (GPC-MALLS) is one of the most reliable and widely used techniques available for the determination of molecular mass and size of polymers over a very wide range. It principally separates molecules based on their hydrodynamic volume ( $V_h$ ) in a column matrix which allows smaller molecules to diffuse in and out of its pores while larger molecules are excluded and pass straight through the column. Its primary use is in measuring molecular weight and molecular weight distributions. The eluting species are then monitored using the MALLS, Refractive Index (RI) and Ultra Violet (UV) detectors.

The MALLS enables the determination of the absolute molar mass of the polymer while the RI detector provides an accurate concentration profile of the eluting species. The UV profile is based on the concentration as well as the chemical nature of the polymer and therefore, detects the proteinaceous materials present in the sample.

Molar mass is obtained by GPC light scattering to characterise molecules with a wide range of particle sizes using the scattered intensity of the molecules at a given scattering angle, thus measurements were made at angles  $>0^\circ$ . Light of a uniform wavelength is discharged from a source, and passes through a

glass cell containing the solution of interest. The Multi angle detectors are positioned at different angles to simultaneously measure the intensity of the scattered light.



**Figure 2.1.** Schematic illustration of light scattering path through polymer solution by (Wyatt Technology Instrument ([www.wyatt.com](http://www.wyatt.com))). Where  $I$  = Incident light and  $\theta$  = scattering angle. (Retrieved from [www.wyatt.com/solutions/sec-mals.html](http://www.wyatt.com/solutions/sec-mals.html) on 19.06.15.)

The polarity of the molecule determines the scattering intensity of the light which is determined by measuring the refractive index increment  $[\text{change in refractive index}]/[\text{change in molecular concentration}] = (dn/dc)$ . Therefore, the intensity of the scattered

light is proportional to the concentration of the macromolecules in solution.

The intensity at  $0^\circ$  is estimated by extrapolation from the higher angle data using a Debye plot. Data from the GPC was analysed using the ASTRA software 4.90.80 package (Wyatt Technology). The Debye plots were constructed using the Zimm method to evaluate data points and to exclude those points that deviated before extrapolation using the Berry method with linear fit (First order polynomial) so as to minimise the effect of possible error in low angle data (Ratcliffe, et al., 2005; Anderson, et al., 2003).

#### **2.2.2.2. Preparation of reagent/eluent**

The solution used during the GPC analysis which serves as the medium (eluent) in the system, is an aqueous 0.1 M sodium nitrate ( $\text{NaNO}_3$ ). The sodium nitrate (Analytical grade) was obtained from Sigma-Aldrich, Gillingham, UK. The eluent was prepared by dissolving 8.5 g of  $\text{NaNO}_3$  in 1000 mL of standard deionised water with the addition of 1 mL of 5% (w/w) sodium azide, which serves as a biocide; the resultant solution was shaken slightly to ensure complete dissolution, and then filtered using a 0.22  $\mu\text{m}$  Phenex nylon membrane filter into a volumetric flask.

#### **2.2.2.3. Preparation of sample for GPC analysis**

As mentioned previously, the gum Arabic samples used in these experiments was in the form of raw, dried, untreated nodules form. These were carefully hand-picked, selected and extraneous materials such as sand and plant bark were removed. The samples were then ground to a fine powder, using a pestle and mortar and sieved using a bin of Mesh size 250  $\mu\text{m}$  so as to obtain a fine and uniform sample.

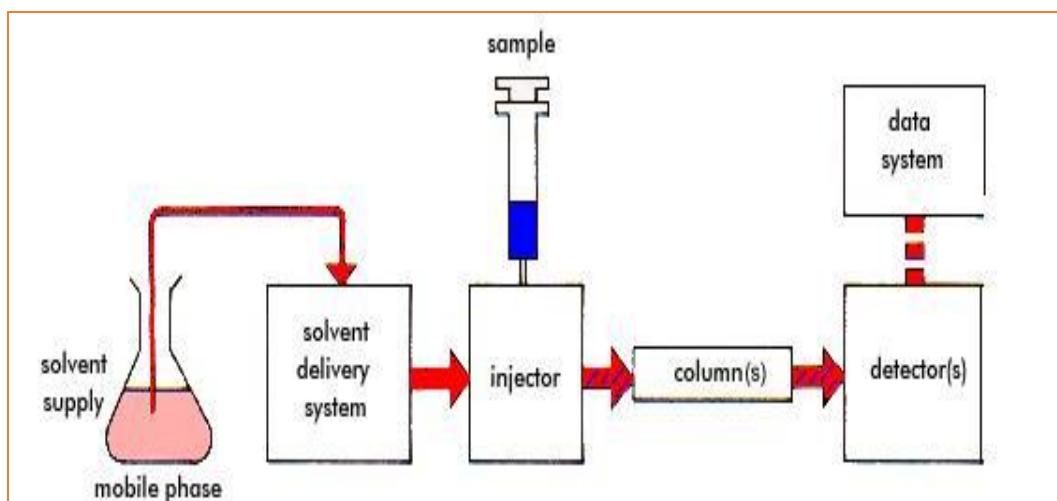
A 0.1 g portion of each sample was then accurately weighed and dispensed into a clean stoppered glass bottle containing a 10 mL solution of 0.1 M sodium nitrate, containing 0.005 % sodium azide and tightly covered. The gum solutions were then placed on a roller mixer at room temperature, until the sample had fully dissolved (approximately 2 hours).

#### **2.2.2.4. Determination of molecular mass distribution**

GPC measures molar mass directly in solution and when combined with a fractionation technique like MALLS, determines absolute molar mass distributions, independent of elution time. The molar mass distribution for each gum sample was obtained using GPC. The system consisted of a Suprema 3000A column of 300 mm x 8 mm dimension and with a bead size of 10  $\mu\text{m}$  and a guard column (Polymer standard service GMBH, Germany); HPLC Pump, (Waters corp. model P-500) set at 0.5 mL/min connected to a rheodyne

injector system (series 7125) with a 200  $\mu\text{m}$  loop volume. The 0.1 M  $\text{NaNO}_3$  (prepared earlier) was used as eluent and was passed through a vacuum degasser (type 006150/4 Cambridge Scientific instruments, U.K.) prior to the rheodyne. A DAWN DSP laser light scattering photometer (Wyatt Technology), Optilab DSP interferometric refractometer (Wyatt Technology Corporation, Santa Barbara, CA, USA) and an Agilent 1100 series (G1314A Agilent Technology) UV-spectrophotometer, set at a wavelength of 280 nm, were used as the detectors. The refractive index increment ( $dn/dc$ ) value used was 0.141 mL/g for *Acacia* gums (Randall, Phillips and Williams, 1989). All samples were filtered through 0.45  $\mu\text{m}$  nylon filters prior to injection into the rheodyne. Data was captured using the designated Astra software for windows (4.90.80, QELSS XX, Wyatt Technology) and then fitted and analysed using the first-order polynomial and the Zimm model.





**Figure 2.2.** Schematic representation of the GPC system showing the basic steps involve in the experiment. The mobile phase contain the eluent that is responsible for transferring/carrying the sample to the detectors for analysis in the column matrix, when it meets the sample at the injector.

#### **2.2.2.5. Moisture content**

To measure the moisture content of each of the samples, 1g of each was placed in a crucible and dried at 105 °C overnight using a Sanyo Convection Oven, Model MOV 212F, Japan. The samples were then transferred to a desiccator and cooled overnight at room temperature to obtain the moisture content. This analysis was performed in triplicate and the average value recorded.

#### **2.2.2.6. Sugar composition**

The sugar compositions of the four gum samples were determined by high performance anion exchange chromatography with pulse

amperometric detection (HPAEC-PAD), using methanolysis combined with trifluoroacetic acid (TFA) hydrolysis using a methodology described previously (Yadav, et al., 2007) with some minor modifications. In brief, the samples were first dissolved in deionized water (1 mg/mL). An aliquot of 100 nmoles myo-inositol (internal standard) was then added to the gum solution and the mixture dried in a Teflon-lined screw cap glass vial, by using filtered nitrogen gas, followed by drying in a vacuum oven at 50 °C overnight. The samples were then methanolized with 1.5M methanolic HCl, in the presence of 20% (v/v) methyl acetate for 16 hours, cooled to room temperature and dried by blowing with filtered nitrogen after the addition of five drops of t-butanol. The methanolized samples were subsequently hydrolyzed with 0.5 ml 2 M Trifluoroacetic acid (TFA) at 121 °C for 1 h, evaporated by blowing with filtered nitrogen at 50 °C and the residue was then washed by sequential addition and evaporation of three aliquots (0.5 mL) of methanol. Into five separate glass vials, were placed 100, 200, 300, 500 and 1000 nmoles of a mixture of standard sugars containing fructose, arabinose, rhamnose, galactose, glucose, xylose, glucuronic acid and galacturonic acid. After which, 100 nmoles of myo inositol (internal standard) was added to each vial, evaporated and dried as above. These standard samples were also methanolized and hydrolyzed as described above, and used for quantification.

The resultant hydrolysates were then analyzed for neutral and acidic sugars by HPAEC-PAD, using a Dionex DX-500 system that included a CarboPac PA20 column and guard column, a GP 50 gradient pump, an ED40 electrochemical detector utilizing the quadruple potential waveform (gold working electrode and pH reference electrode), an AS3500 auto sampler with a thermal compartment (30 °C column-heater), and a PC10 pneumatic controller post column addition system. The mobile phase consisted of isocratic 12 mM NaOH eluant for 10 min followed by 100 mM NaOH and 6 mM CH<sub>3</sub>COONa for 3 min, 100 mM NaOH and 12 mM CH<sub>3</sub>COONa for 17 minutes at a flow rate of 0.5 mL/min. at ambient temperature. The column was washed with 1M CH<sub>3</sub>COONa for 0.10 min and with 100 mM NaOH for 10 minutes, followed by 30 min equilibration with 12 mM NaOH at a flow rate of 0.5 mL/min at ambient temperature was required to yield highly reproducible retention times for the monosaccharides. The total run time was about 70 minutes. In order to minimize baseline distortion due to changes in the pH of the eluant during monosaccharide detection (by PAD) 730 mM NaOH was added to the post column effluent via a mixing tee.

#### **2.2.2.7. Glucuronic Acid Content**

The glucuronic acid content of the samples was determined using a modified version of the method for quantitative determination of

uronic acid (Blumenkrantz and Asboe-Hansen, 1973). Glucuronic acid, meta-hydroxydiphenyl, sodium tetraborate, concentrated sulphuric acid and sodium hydroxide were obtained from Fisher Scientific. A 0.15% solution of meta-hydroxydiphenyl in 0.5% sodium hydroxide and a 0.0125 M solution of sodium tetraborate in concentrated sulphuric acid were prepared. A calibration curve was initially determined using glucuronic acid. 0.4 mL solutions containing 0.5 to 20  $\mu$ L glucuronic acid were pipetted into screw cap glass tubes and 2.4 mL of sulphuric acid/sodium tetraborate reagent was added and screw caps fitted. The tubes were then heated in a water bath at 100 °C for a period of exactly 5 minutes. The samples were then cooled immediately in a water ice bath for 5 minutes and 40  $\mu$ L of meta-hydroxydiphenyl reagent was then added. The tubes were shaken slightly and the solution immediately transferred into a quartz cuvettes (spectroscopy experiment grade) 10 mm path length (CXA-110-0053 from Fishes Scientific Ltd, UK.) and the absorbance at 520 nm was measured (all within 5 minutes) using a Lambda 25 UV/VIS spectrophotometer (Perkin Elmer).

A blank run was then performed without the addition of meta-hydroxydiphenyl reagent, which was replaced with 40  $\mu$ L of 0.5% sodium hydroxide. This blank run was performed since the other sugars present in gum Arabic produce a pinkish chromogen with sulphuric acid/tetraborate at 100 °C. The absorbance for the blank

sample was subtracted from the original absorbance and the glucuronic acid content was thus determined from the calibration curve.

#### **2.2.2.8. Protein content**

Protein content analysis was carried out using the AOAC approved Kjeldahl method (AOAC, 1999), a method developed by Danish scientist, Johan Kjeldahl in 1883. This method involves the digestion of the sample with strong sulphuric acid ( $\text{H}_2\text{SO}_4$ ) in the presence of a catalyst (0.4 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) which helps in the conversion of amine nitrogen into ammonium ions ( $\text{NH}_4^+$ ), followed by further conversion of the ammonium ions into ammonia ( $\text{NH}_3$ ) gas which is then released from the solution by steam distillation and condense as ammonium hydroxide ( $\text{NH}_4\text{OH}$ ). This together with the distillate are then collected. The distillate is titrated with 0.1 M HCl in the presence of 2% boric acid to give the total nitrogen content. A nitrogen conversion factor for *Acacia* gums of 6.60 was used ( $\text{N} \times 6.60$ ) as suggested by (Anderson, 1986).

#### **2.2.2.9. Amino acid composition**

Amino acid analysis was performed by Alta Bioscience, University of Birmingham, U.K. The gum Arabic samples were first hydrolysed in a special vacuum hydrolysis tube, in which accurately weighed samples were dissolved with 6 mol  $\text{dm}^{-3}$  HCl of known volume (1

cm<sup>3</sup>). A custom designed and built, automatic amino acid analyser was then used to measure the amino acids in the samples, which was based on modular HPLC components controlled by a Nascom III computer. Each sample was injected onto the top of the column containing a strong cation exchange resin, maintained at temperature of 55 °C. The amino acids were separated and eluted off the cation exchange column, in order of their net charge value, using a series of sodium citrate buffers, producing a stepwise gradient with increasing pH and ionic strength. The flow rate of the eluent (citrate buffers) was maintained at 0.22 cm<sup>3</sup>/min using an LKB pump. A Waters WISP sampler was used to sample the eluting amino acids, which were mixed with a colouring reagent (ninhydrin) that was eluted at a rate of 0.3 cm<sup>3</sup>/min using an ACS 400 pump. The colorimetric reaction was carried out at 120 °C for 3 minutes. Then, the absorbance was monitored at 440 and 550 nm using a Waters UV detector. A Linseis recorder was used for recording the chromatogram.

Hydroxyproline, a principal amino acid present in gum Arabic, has a low response factor and was contained within the Aspartic acid peak. The two peaks were resolved by lowering the temperature of the cation exchange column to 40 °C. Detection was carried out at 440 nm and data expressed as the ratio of each amino acid, these were normalised with the initial experimental data by comparing the hydroxyproline/leucine ratios.

#### **2.2.2.10. Molecular association**

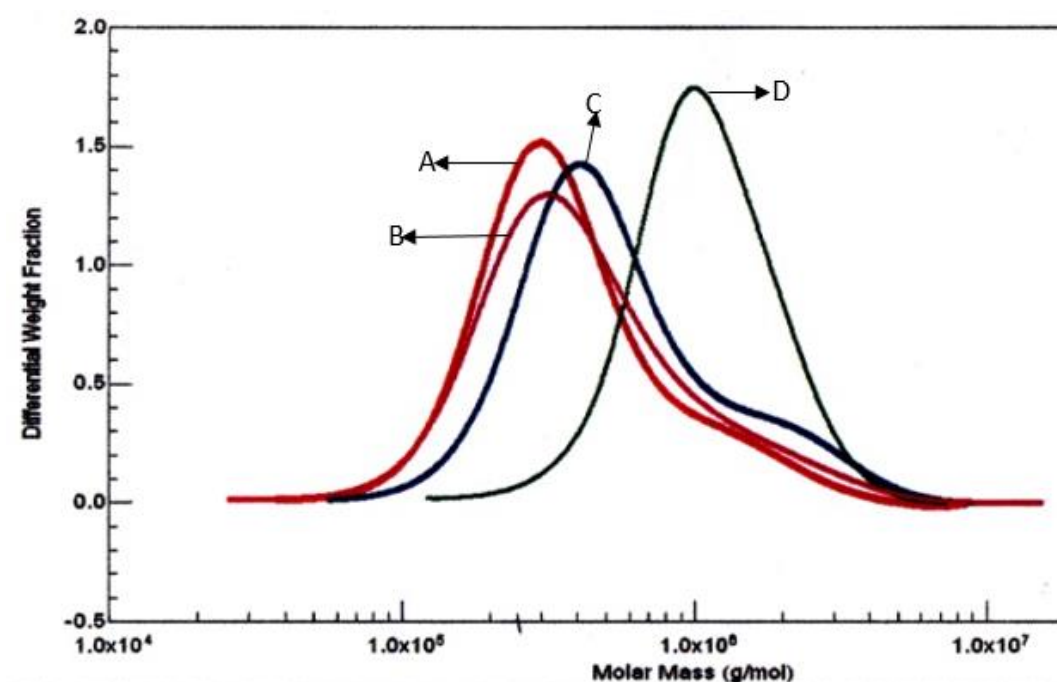
The molecular association of gum Arabic, was investigated by measuring the hydrodynamic size as a function of time, in both water and in 0.5 M sodium nitrate using the Zetasizer Nano series ZS, (Malvern Instruments). Gum Arabic solutions, 10% (w/w) for *Acacia senegal* and *Acacia seyal* samples were prepared in deionized water and in 0.5 M sodium nitrate. The hydrodynamic size was then determined as a function of time.

## **2.3.0. Results**

### **2.3.1. Molecular mass parameters**

The molecular mass distribution profiles of gum Arabic samples obtained by GPC/MALLS and RI detectors is presented in Figure 2.3. Samples A, B and C were found to have similar molecular mass profiles, however, a significant difference in the molecular mass distribution was observed for sample D (A= *A. senegal*-NG<sub>1</sub>; B= *A. senegal*-NG<sub>2</sub>; C= *A. senegal*-Sudan; D= *A. seyal*). The actual values of the individual molecular mass of the samples is as shown in Table 2.1. The molecular mass values are consistent with those previously obtained in the literature and confirm the samples to be *Acacia* gums.





**Figure 2.3.** Molecular mass distribution profiles of gum Arabic samples obtained by GPC/MALLS and RI detectors. (A= *A. senegal*-NG<sub>1</sub>; B= *A. senegal*-NG<sub>2</sub>; C= *A. senegal*-Sudan; D= *A. seyal*). It showed different profiles of the different *Acacia* gumb samples. Sample D (*A. seyal*), exhibits an entirely different profile compared to the remaining *A. senegal* gum samples. Although the three different *Acacia senegal* gum samples (A, B and C) appears to be similar, a slight difference can be observed with the profile of sample C (*A. senegal* from sudan) which could be attributed to some variation in chemical composition and the differences in ecolocation where these gums are produced.

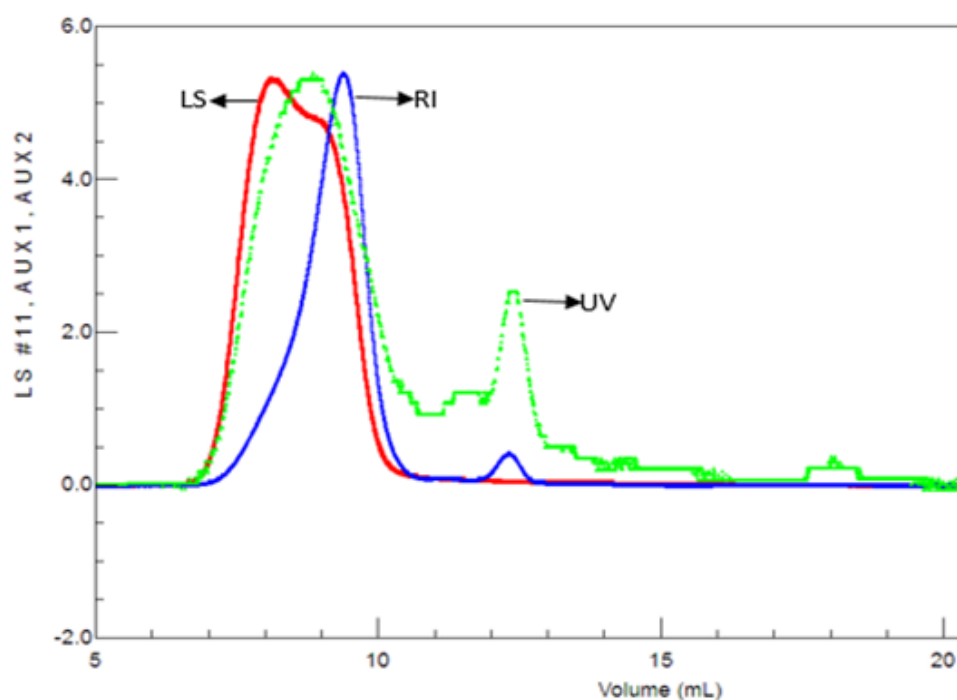
The result of the molar mass parameters are summarised and presented in Table 2.1 showing the molecular mass and the hydrodynamic radius of the four samples.

**Table 2.1.** Molar mass and hydrodynamic size of *Acacia* gum exudates obtained by GPC-MALLS and DLS techniques.

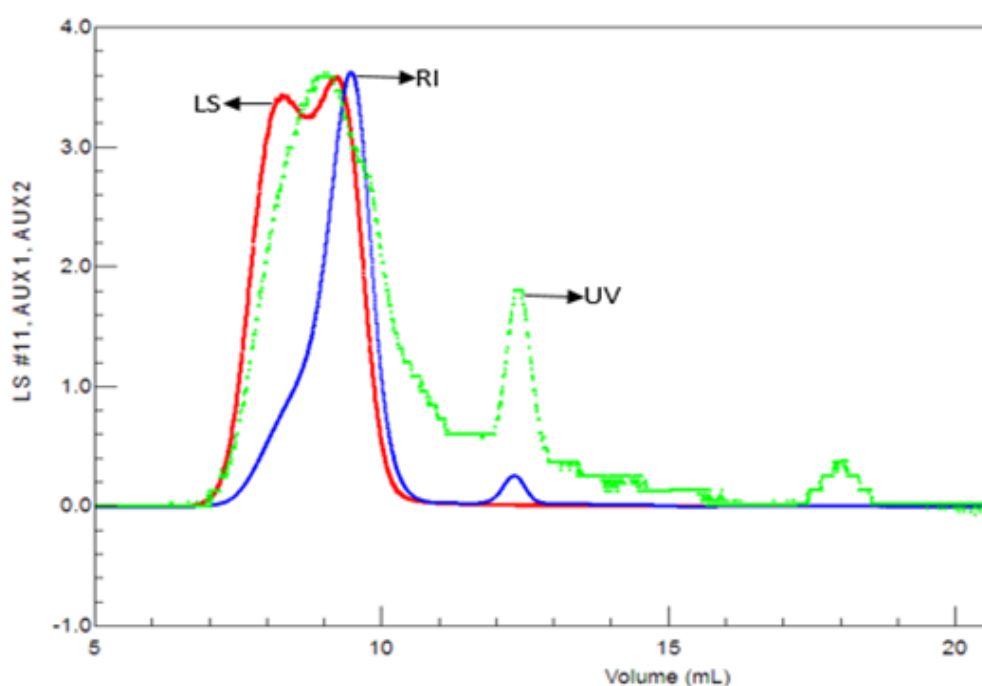
	<i>A. senegal</i> (NG <sub>1</sub> ) A.	<i>A. senegal</i> (NG <sub>2</sub> ) B.	<i>A. senegal</i> (Sudan) C.	<i>A. seyal</i> D.
Mw	4.85 x 10 <sup>5</sup>	7.43 x 10 <sup>5</sup>	6.40 x 10 <sup>5</sup>	1.14 x 10 <sup>6</sup>
Mn	2.77 x 10 <sup>5</sup>	3.94 x 10 <sup>5</sup>	3.52 x 10 <sup>5</sup>	6.84 x 10 <sup>5</sup>
Mw/Mn	1.8	1.9	1.9	1.7
R <sub>g</sub>	13.0	17.9	18.5	24.0
R <sub>h</sub>	13.0	15.4	15.2	17.3
R <sub>g</sub> /R <sub>h</sub>	1.0	1.2	1.2	1.4

Mw=Molecular weight, Mn=Number molecular mass, R<sub>g</sub>=Radius of gyration (R.M.S) (Obtained from GPC-MALLS), R<sub>h</sub>=Hydrodynamic radius (obtained using DLS). The result reflects the observations in the distribution profile. Details of their implications are discussed in the discussion section of this chapter.

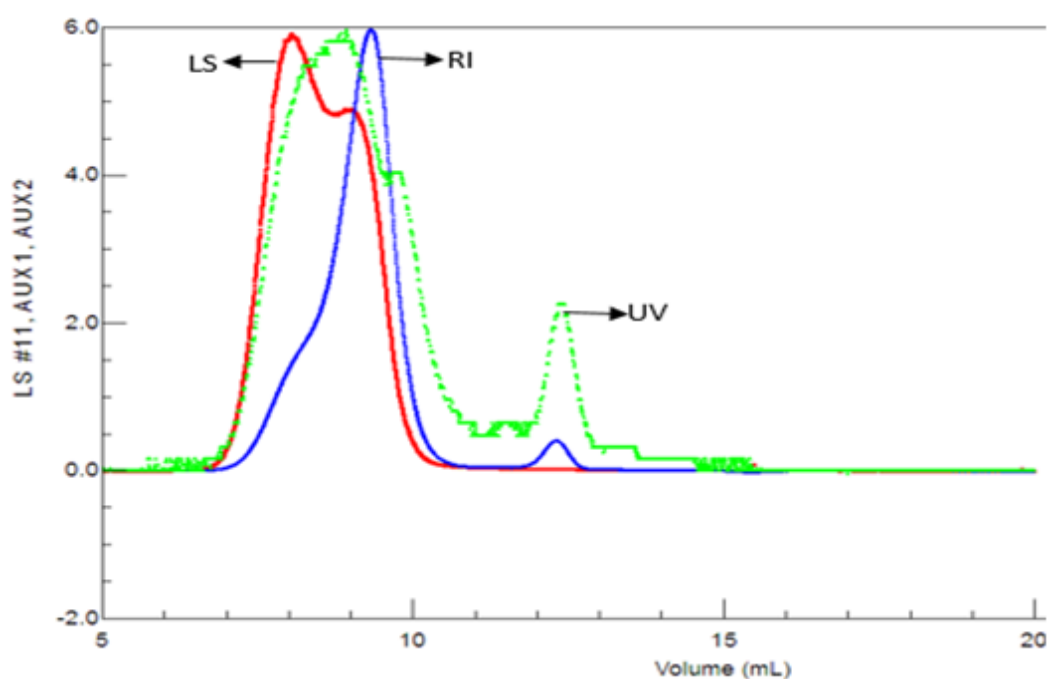
The elution profiles of the four gum samples studied using MALLS, RI and UV (at 280 nm) are presented in Figures 2.4. to 2.7. Light scattering is sensitive to concentration and molecular mass, while the RI is a sensitive measure of concentration of the eluting species. The UV measures the concentration as well as being indicative of the chemical nature of the material, especially the proteinaceous components.



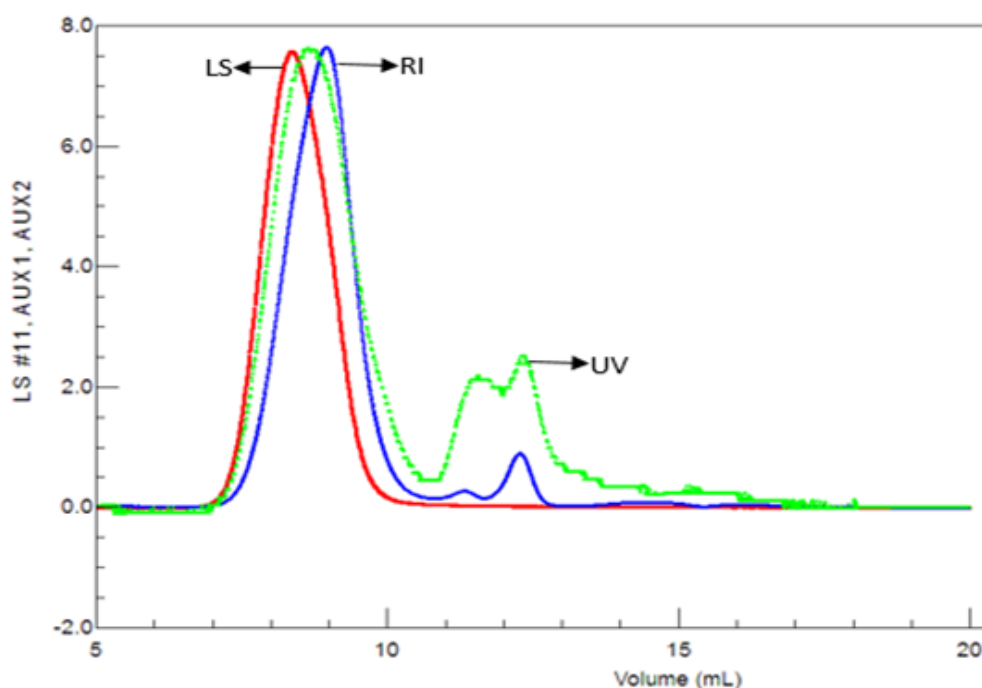
**Figure 2.4.** GPC elution profiles of *A. senegal*-Sudan gum sample using LS/RI/UV detectors. The distribution of sizes of the different molecular species are indicated by the LS, and it can be observed that there are more of the larger size molecules in the sample, as they are eluted from the column first. The RI gives the concentration profile of the eluting species which showed that there is low concentration of the larger molecules eluting at ~8 mL. Most of the molecules are found at ~9 – 10 mL. The UV gives the concentration and chemical nature of the molecules, especially the proteinaceous components. Details of which were discussed later in this chapter. This trend is applicable to figures 2.5, 2.6 and 2.7 below.



**Figure 2.5.** GPC elution profiles of *Acacia senegal*-NG<sub>1</sub> gum sample (B) using LS/RI/UV detectors. The light scattering LS showed the size distribution of the molecules, while the RI showed the concentration profile of the different eluting molecular species. UV at (280 nm) is indicative of the concentration and chemical nature of the gum, specifically the proteinaceous component.

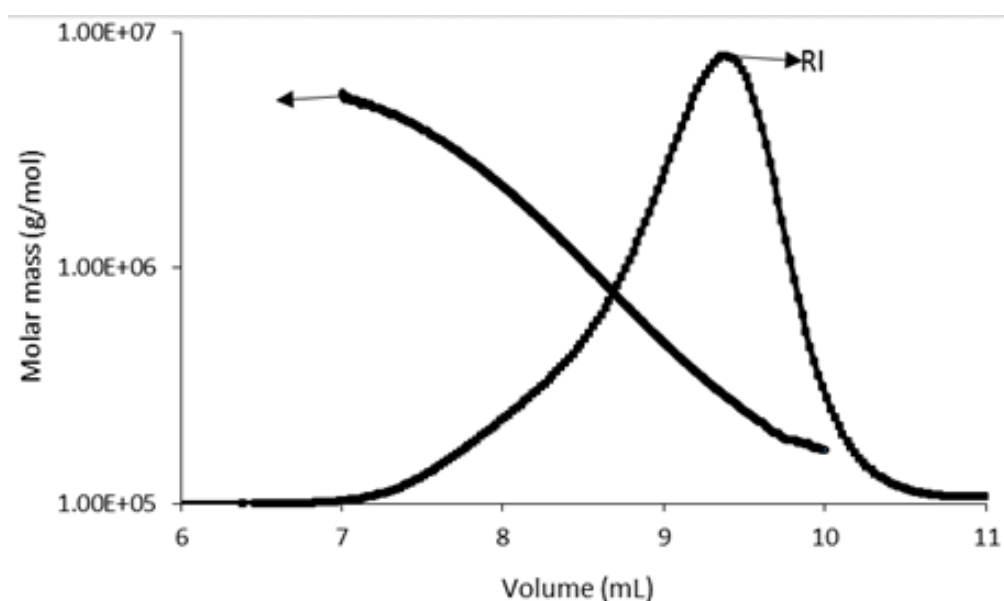


**Figure 2.6.** GPC elution profiles of *Acacia senegal*-NG<sub>2</sub> gum sample using LS/RI/UV. The light scattering LS showed the size distribution of the molecules, while the RI showed the concentration profile of the different eluting molecular species. UV at (280 nm) is indicative of the concentration and chemical nature of the gum, specifically the proteinaceous component.



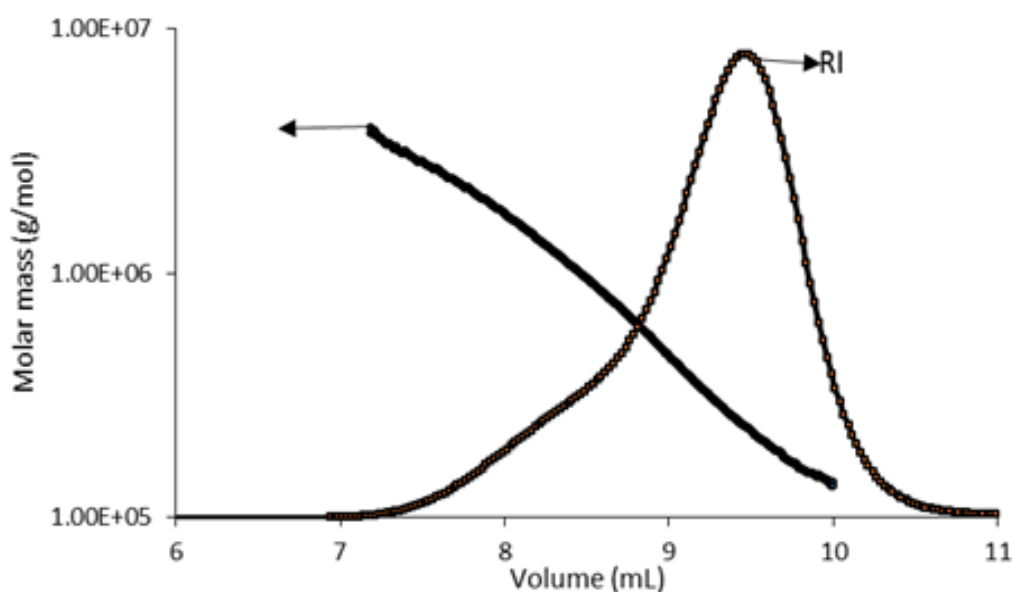
**Figure 2.7.** GPC elution profiles of *Acacia seyal* gum sample using LS/RI/UV detectors. The light scattering LS showed the size distribution of the molecules, while the RI showed the concentration profile of the different eluting molecular species. UV at (280 nm) is indicative of the concentration and chemical nature of the gum, specifically the proteinaceous component.

Figures 2.8 to 2.11 reports the RI/MW profiles and Figures 2.12 to 2.15 are the RI/R.M.S profiles for the gum samples as a function of elution volume determined using the Astra software designed and accompanied with the specific GPC model.

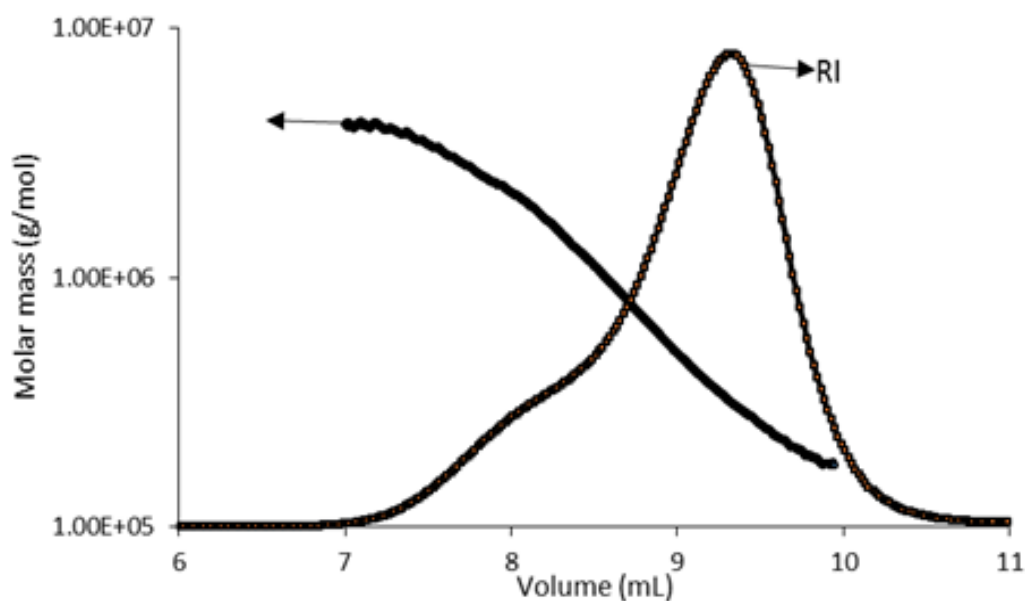


**Figure 2.8.** RI/Mw profile for *Acacia senegal*-Sudan gum sample. The RI and Mw elution profiles for this *Acacia* gum sample shows that, the Mw for the AGP and AG fraction eluted at different peak maximum, ranging from 8.0 - 9 mL, and yield different molar masses for the two molecular species.



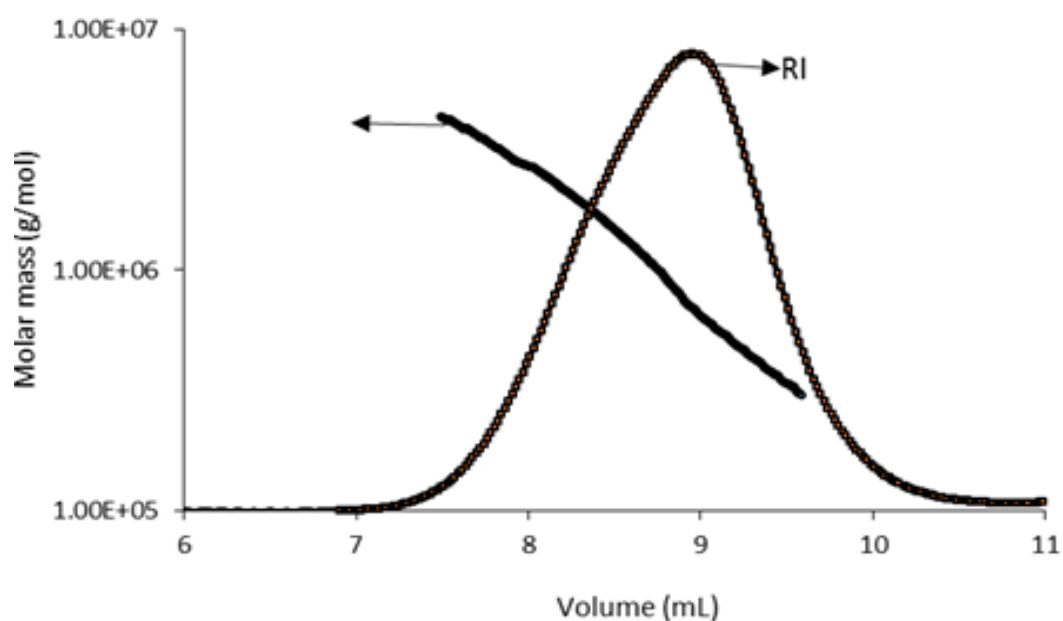


**Figure 2.9.** RI/Mw profile for *Acacia senegal*-NG<sub>1</sub> gum sample. The RI and Mw elution profiles for the *Acacia* gum samples show that, the Mw for the AGP and AG fraction also eluted at different peak maximum ranging from 8.0 - 9 mL, and yield different molar masses for the two molecular species as observed in the *Acacia senegal*-Sudan sample.

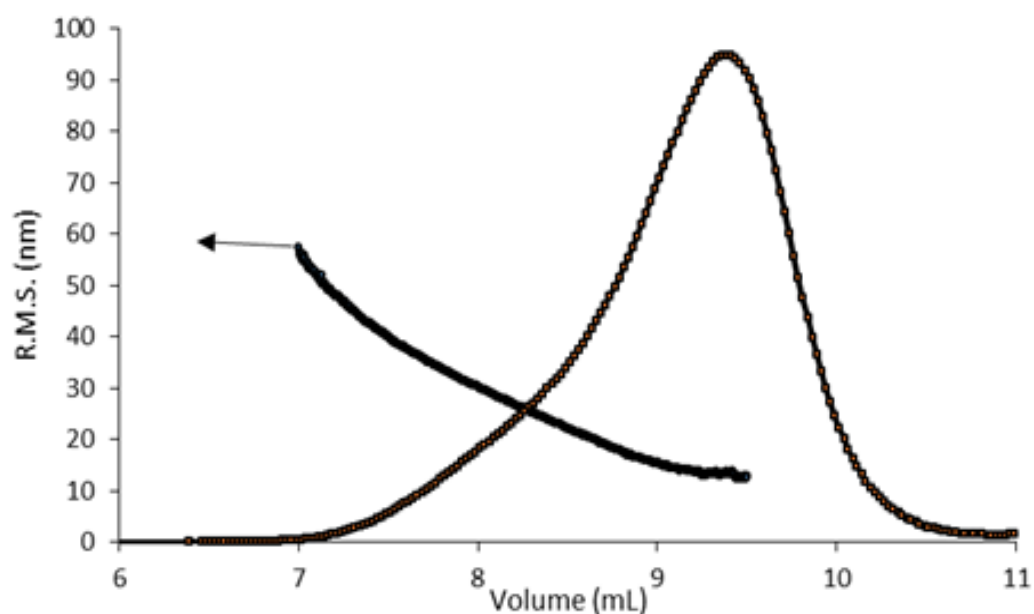


**Figure 2.10.** RI/Mw profile for *Acacia senegal*-NG<sub>2</sub> gum sample.

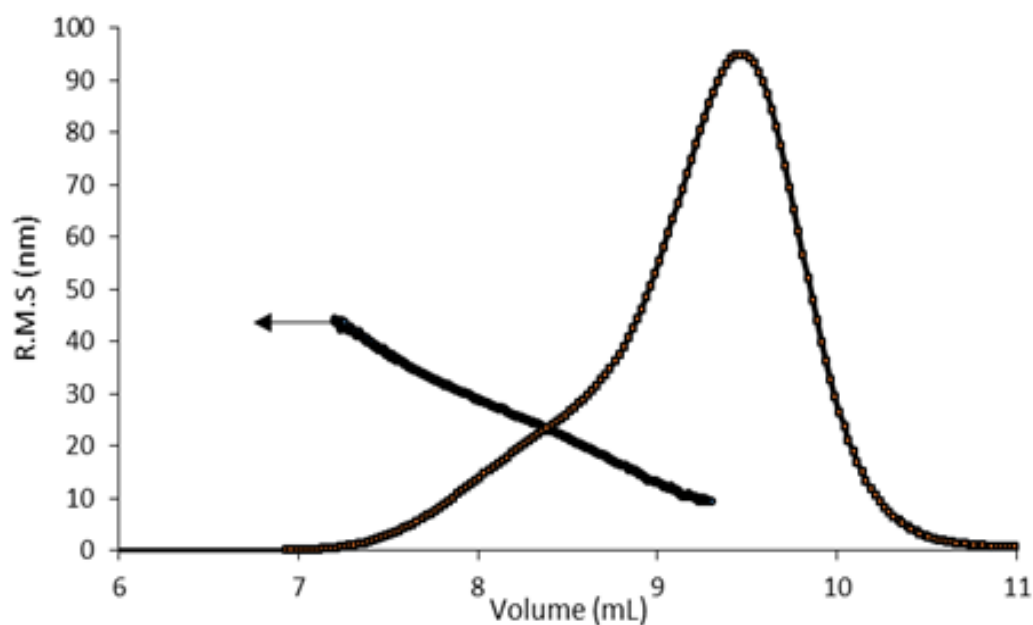
The RI and Mw elution profiles for the *Acacia* gum samples show that, the Mw for the AGP and AG fractions eluted at different peak maximum ranging from 8.0 – 9.0 mL, and yield different molar masses for the two molecular species. This sample also showed closer similarity to the *Acacia senegal*-Sudan and *Acacia senegal*-NG<sub>1</sub> sample in terms of their elution profiles, but in all the three samples, the value of their molar mass were different.



**Figure 2.11.** RI/Mw profile for *Acacia seyal* gum sample. In the *A. seyal* sample there is no observed peak for the AGP fraction, but the Mw for the AG fraction at the peak maximum (9.0 mL) is ~600 kDa. An almost uniform elution pattern was observed, indicating very little presence of the AGP component.

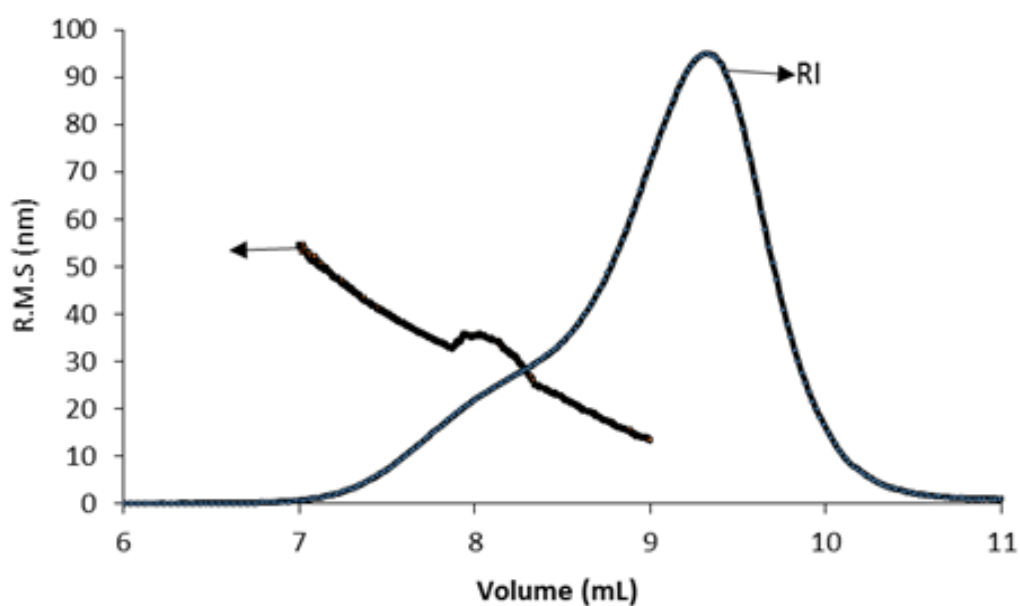


**Figure 2.12.** RI/Radius profile of *Acacia senegal*-Sudan gum sample. The RI and  $R_g$  elution profiles for this samples showed that the  $R_g$  for the AGP component at the peak maximum of 8.5 mL is  $\sim 25$  nm. It is not possible to determine the  $R_g$  at the peak maximum for the AG fraction since the values are too small, i.e.  $< 10$  nm which is beyond the limits of the technique used.



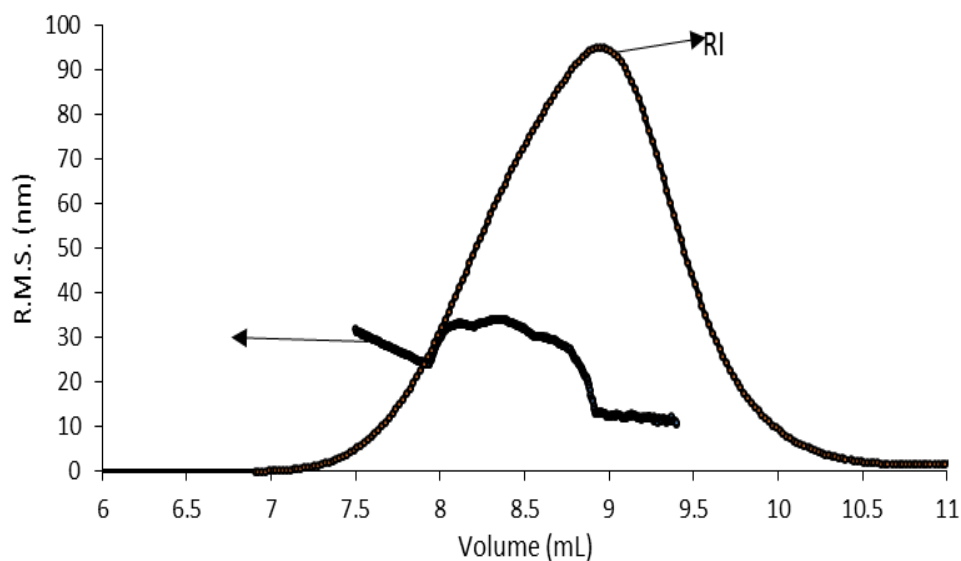
**Figure 2.13.** RI/Radius profile of *Acacia senegal*-NG<sub>1</sub> gum sample.

The RI and  $R_g$  elution profiles for this samples also showed that the  $R_g$  for the AGP component at the peak maximum of 8.5 mL is  $\sim 25$  nm. It is not possible to determine the  $R_g$  at the peak maximum for the AG fraction since the values are too small, i.e.  $< 10$  nm which is beyond the limits of the technique used. This sample showed similar  $R_g$  profile to the *Acacia senegal*-Sudan.



**Figure 2.14.** RI/Radius profile of *Acacia senegal*-NG<sub>2</sub> gum sample.

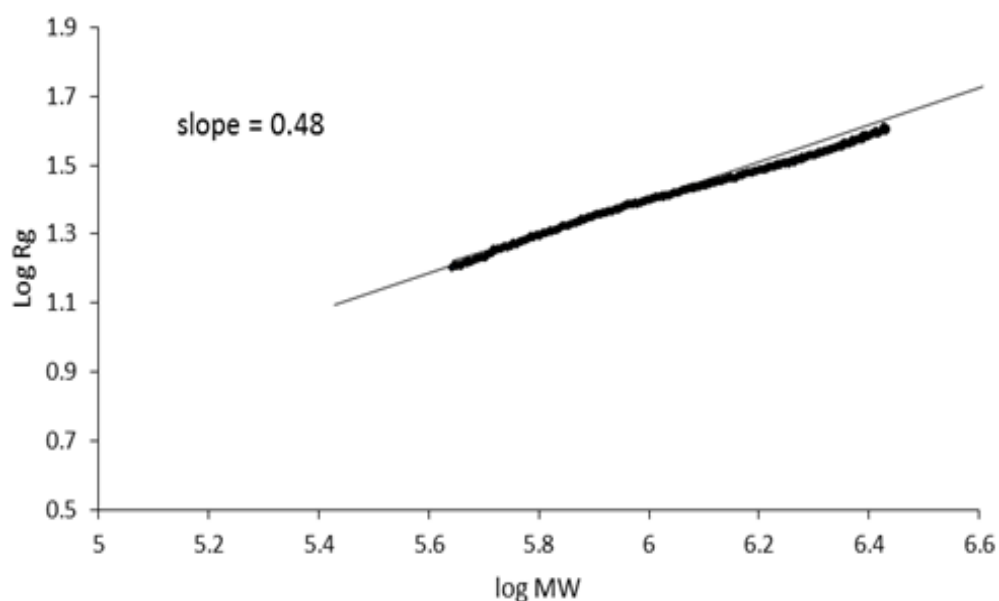
The  $R_g$  profile for the *A. senegal*-NG<sub>2</sub> sample showed an unexpected distortion in the line at an elution volume of  $\sim 8.0$  mL corresponding to the AGP.



**Figure 2.15.** RI/Radius profile of *Acacia seyal* gum sample. Interestingly, the  $R_g$  profile for the *A. seyal* sample also shows a distortion in the line. This observation cannot be explained at this point, other than to say that the distortion occurs at elution volumes corresponding to the AGP fraction indicating a different molecular structure.

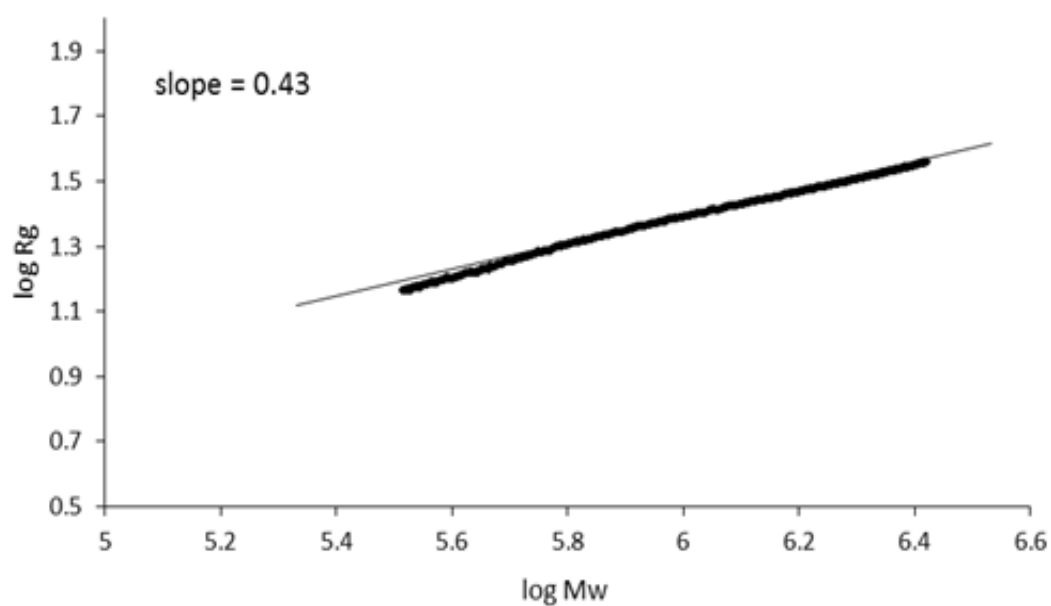
A plot of log Root Mean Square (RMS) radius of gyration ( $R_g$ ) versus log molecular weight ( $M_w$ ) has been known to reveal useful information about the conformation of polymer solution (Schittenhelm and Kulicke, 2000).

The slope gives an indication about the conformation of the polymer molecules, which could be in the form of spheres, random coils or rigid rods (William and Langdon, 1995). For this work, the plots for all the samples using the  $\log R_g$  versus  $\log M_w$  plots are presented in figures 2.6 - 2.9 below.

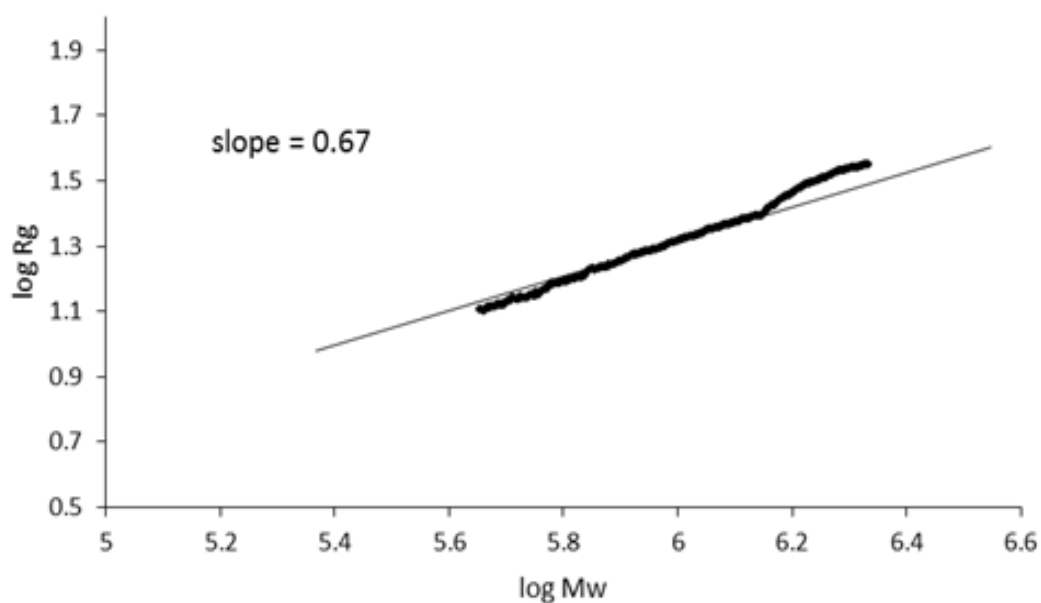


**Figure 2.16.**  $\log R_g$  against  $\log M_w$  of *Acacia senegal*-Sudan gum sample. The slope is linear and gives a value of 0.43 which determine the structure of the molecule, and is consistent with a highly branched compact molecular structure.

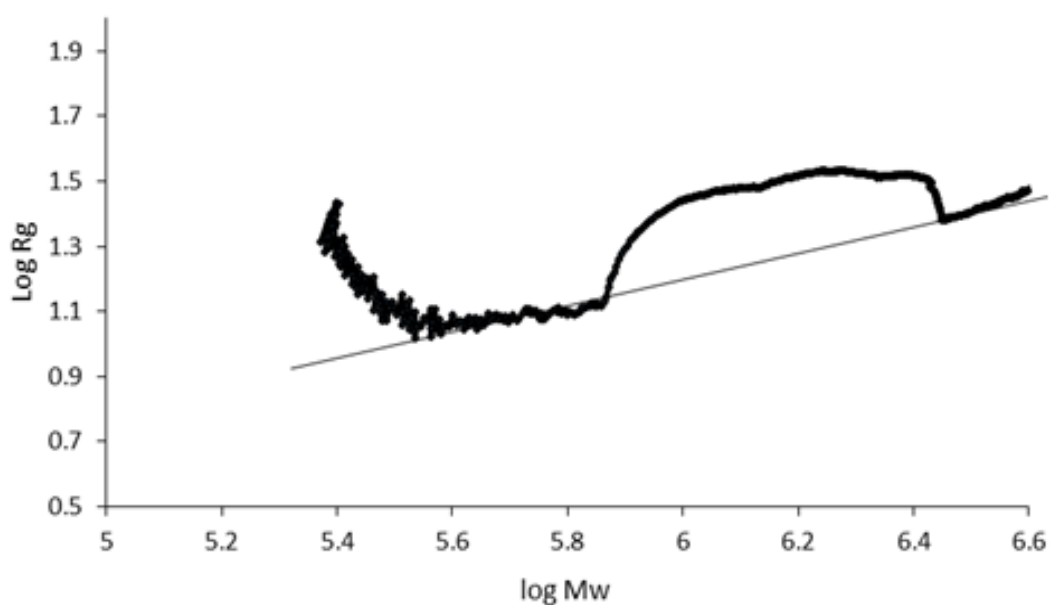




**Figure 2.17.**  $\log R_g$  against  $\log M_w$  of *Acacia senegal*-NG<sub>1</sub> gum sample. The plot of  $\log R_g$  against  $\log M_w$  for the *Acacia senegal*-NG<sub>1</sub> gum sample also gives a linear slope but with a value of 0.46. This is also consistent with a highly branched compact molecular structure. These two samples exhibit similar molecular structure.



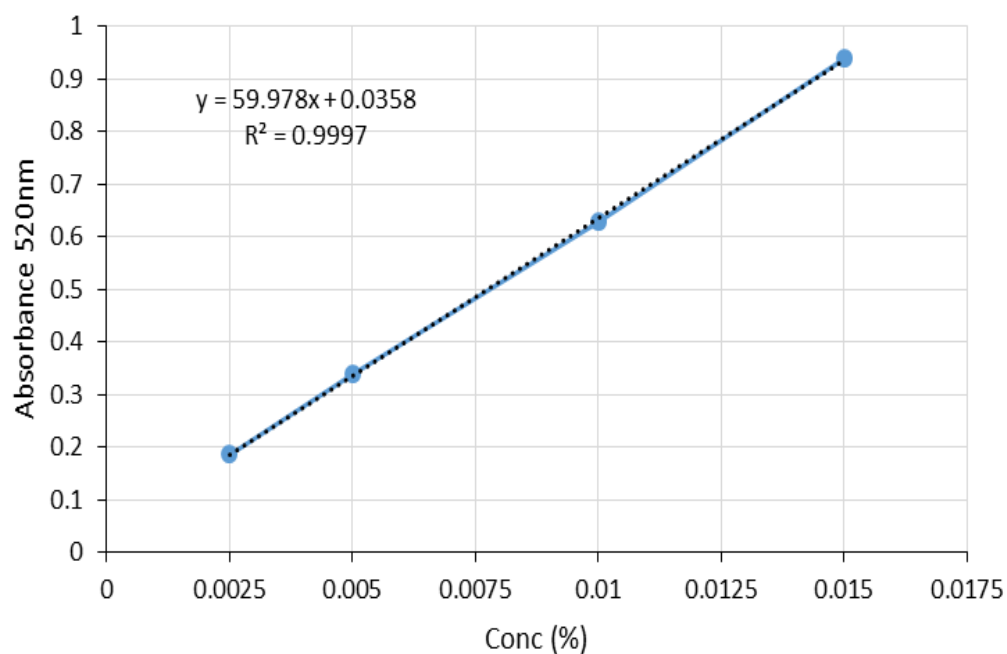
**Figure 2.18.**  $\log R_g$  against  $\log M_w$  of *Acacia senegal*-NG<sub>2</sub> gum sample gave a comparatively higher value 0.67, as compared to the other two gum samples presented above. The significantly higher value for the slope of this sample indicates some structural differences.



**Figure 2.19.**  $\log R_g$  against  $\log M_w$  of *Acacia seyal* gum sample. The plot for this gum sample was not linear and a meaningful value for the slope could not be obtained from the equation for determination of the slope.

### **2.3.2. Sugar composition and protein content**

As mentioned in the methodology, the sugar composition of the samples was determined by HPAEC-PAD, using methanolysis combined with TFA hydrolysis and the result presented in Table 2.2 below. All the four samples were found to contain arabinose, rhamnose, galactose and glucuronic acid, with the *A. seyal* having less rhamnose compared to all the *A. senegal* samples. The protein content (determined using the kjeldahl method and from the amino acid composition) are also presented in the same Table. The glucuronic acid was measured after a standard calibration curve was first determined.



**Figure 2.20.** Standard calibration curve used for the determination of glucuronic acid content using the modified method of Blumenkrantz and Asboe-Hansen, (1973) for the quantitative determination of uronic acid. (Linear regression equation:  $y = 59.978x + 0.0358$ ;  $R^2 = 0.9997$ ).

**Table 2.2.** Sugar composition and protein content of *Acacia* gum exudates (% w/w) on dry weight basis

	<i>A. senegal</i> (Sudan)	<i>A. senegal</i> (NG <sub>1</sub> )	<i>A. senegal</i> (NG <sub>2</sub> )	<i>A. seyal</i>
Rhamnose	9.5	9.2	7.7	2.0
Arabinose	25.7	24.9	23.6	32.5
Galactose	38.9	45.3	38.8	44.2
Glucuronic acid	21.5	15.5	20.2	13.0
Total sugar (%)	95.6	94.9	90.1	91.7
Protein <sup>a</sup>	2.09	2.93	2.66	0.99
Protein <sup>b</sup>	1.69	2.12	2.13	0.69

<sup>a</sup>=From Kjeldhal method using NCF 6.60 as suggested by Anderson, (1986), <sup>b</sup>=From amino acid analysis.

Significant differences were observed between the monosaccharide sugar composition of the *A. senegal* and *A. seyal* gum samples, with the *A. seyal* gum sample having very low rhamnose and glucuronic acid and high arabinose content. Low glucuronic acid and high galactose was observed in the *A. senegal*-NG<sub>1</sub> compared to the other two *A. senegal* samples. The *A. seyal* has the least protein content while the *A. senegal*-NG<sub>1</sub> contains the highest content of all the samples and twice as much compared to the *A. seyal* gum sample.

### **2.3.3. Amino acid composition**

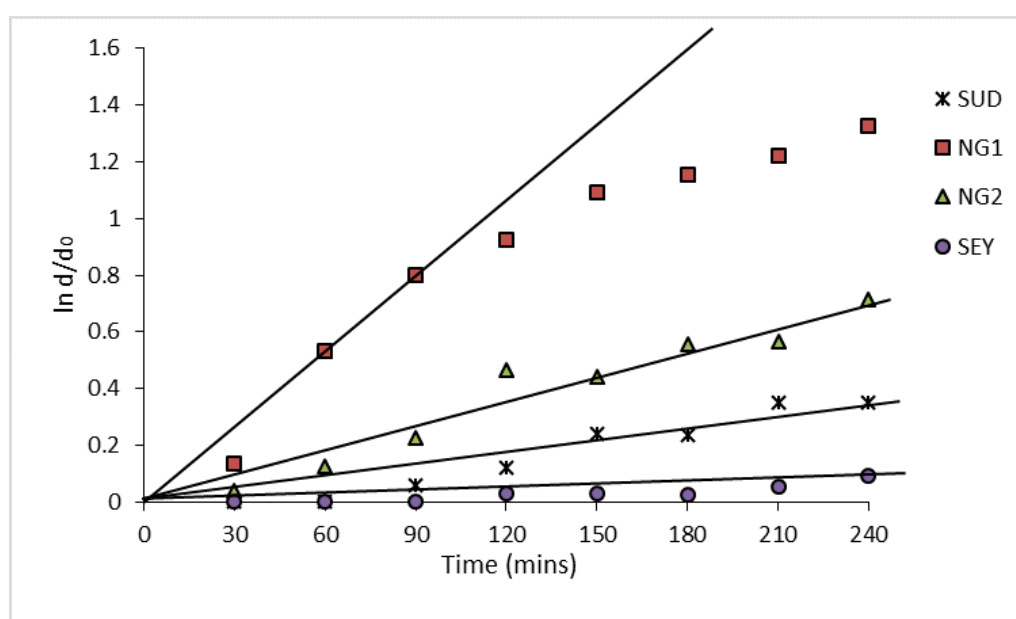
The amino acid composition of the samples was analysed using an hydrolysis method based on modular HPLC components. The results of which are presented in Table 2.3. The main amino acids detected were shown to be Hydroxyproline, Serine, Leucine, Threonine, Histidine and Aspartic acid. *A. senegal*-NG<sub>1</sub> has the highest amount of total amino acid while the *A. seyal* had the least of all the samples.

**Table 2.3.** Amino acid composition of *Acacia* gum exudates (g/100 g) on a dry weight basis

	<i>A. senegal</i> (Sudan)	<i>A. senegal</i> (NG <sub>1</sub> )	<i>A. senegal</i> (NG <sub>2</sub> )	<i>A. seyal</i>
Hydroxy-proline	0.453	0.521	0.476	0.201
Aspartic acid	0.104	0.144	0.167	0.041
Threonine	0.139	0.170	0.168	0.046
Serine	0.217	0.278	0.267	0.100
Glutamic acid	0.073	0.109	0.136	0.026
Proline	0.114	0.140	0.140	0.053
Glycine	0.050	0.063	0.066	0.016
Alanine	0.030	0.040	0.044	0.016
Valine	0.058	0.081	0.092	0.034
Methionine	0.002	0.003	0.002	0.001
Isoleucine	0.024	0.030	0.027	0.018
Leucine	0.139	0.170	0.174	0.053
Tyrosine	0.019	0.023	0.017	0.010
Phenyl-alanine	0.075	0.016	0.130	0.021
Histidine	0.124	0.143	0.135	0.044
Lysine	0.051	0.070	0.069	0.012
Arginine	0.021	0.027	0.025	0.009

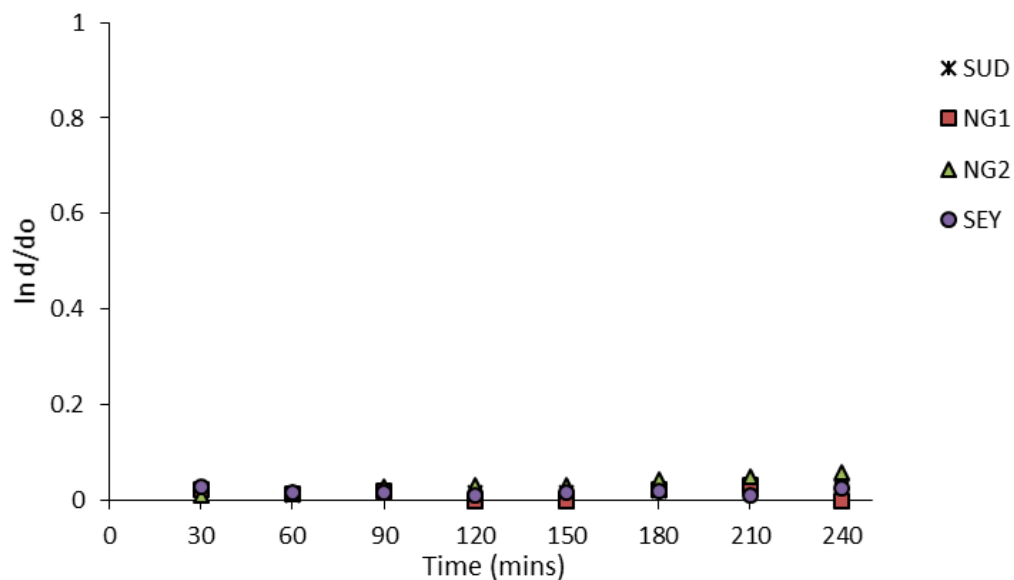
### 2.3.4. Molecular association in solution

The association of the gum Arabic samples in solution was studied in water and in 0.5 M NaNO<sub>3</sub> and the results are presented in Figures 2.21 - 2.23. A significant change was observed in the behaviour of the gums under salt condition as compared to the presence of water.

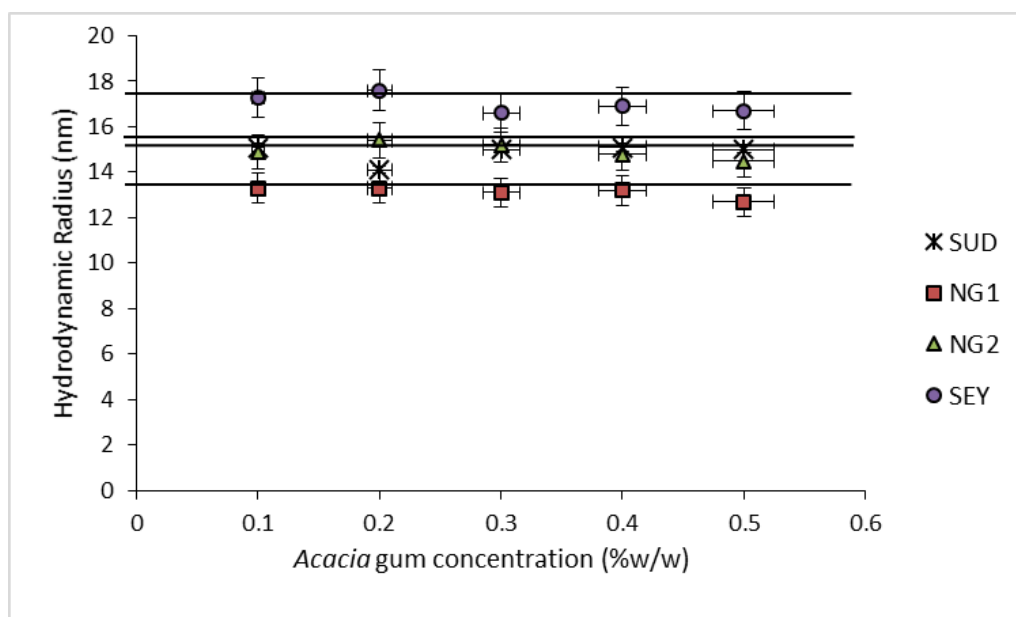


**Figure 2.21.**  $\ln d/d_0$  of 10% (w/w) solutions of *Acacia* gums in water as a function of time. ( $\ln$ = natural log,  $d_0$ = initial diameter and  $d$ = final diameter. The trend line indicates rate constant.). The hydrodynamic diameter of the different *Acacia* gum samples was determined in water over a period of 240 minutes to determine any increment. The *Acacia senegal*-NG<sub>1</sub> was observed to significantly increase with time while the *A. seyal* showed little increase over the observed period.





**Figure 2.22.**  $\ln d/d_0$  as a function of time for 10% solutions of *Acacia* gums in 0.5 M  $\text{NaNO}_3$ . The hydrodynamic diameter of the different *Acacia* gum samples was determined in 0.5 M  $\text{NaNO}_3$  over a period of 240 minutes to determine any change in the behaviour of the molecules of the gum samples. All the samples showed no increase in their hydrodynamic diameter with time over the observed period.



**Figure 2.23.** Hydrodynamic radius of *Acacia* gums as a function of concentration in 0.5 M NaNO<sub>3</sub>. The z-average hydrodynamic radius,  $R_h$ , of the gum samples was determined in the presence of 0.5 M NaNO<sub>3</sub> as a function of their concentration.  $R_h$  value was obtained by extrapolation to zero concentration. The *A. seyal* gum sample was observed to have a higher hydrodynamic radius compared to all the other three *A. senegal* gum samples.

## **2.4.0. Discussion**

### **2.4.1. Molecular mass parameters**

The weight average ( $M_w$ ) and number average ( $M_n$ ) molecular mass values obtained by GPC are given in Table 2.1. The values for the Sudanese and Nigerian *A. senegal* gum samples, are both of the same order of magnitude and are significantly lower than the values obtained for the gum sample obtained from *A. seyal*. The average molecular weight of the gum collected from *A. seyal* has been previously reported to be higher than that of *A. senegal* and that *A. seyal* is more highly branched and more compact in structure than *A. senegal* (Williams and Phillips, 2000; Hassan, et al., 2005). These results are consistent with the literature, which also shows, as mentioned previously, that such values can vary depending upon the precise molecular structure/composition of the gum, which is affected by an excess of biotic and abiotic factors (Idris, Williams and Phillips, 1998; Siddig, et al., 2005; Al-Assaf, Phillips and Williams, 2005b; Renard, et al., 2006).

Light scattering is a non-destructive method for characterizing molecules and a wide range of particles in solution. The intensity of the scattered light is measured as a function of angle. In the case of macromolecules, this is often called Rayleigh scattering and can yield the molar mass, root mean square radius, and second virial coefficient ( $A_2$ ). It is also applied in the determination of size, shape, and structure. The GPC elution profiles for the three *A.*

*senegal* gum samples presented in Figures 2.4, 2.5 and 2.6 showed that, the RI profile included a large peak, with a peak maximum at  $\sim 9.4$  mL, together with a small shoulder on the high molecular mass side. The main peak is attributed to the arabinogalactan (AG) fraction and the shoulder is attributed to the arabinogalactan protein (AGP) fraction as reported previously (Randall, Phillips and Williams, 1989). The small peak at  $\sim 12.5$  mL is the salt peak, ( $V_t$ ). The UV elution profiles are significantly different to the RI profiles due to the fact that UV is sensitive not only to the concentration of the eluting material, but also to its chemical nature. It is expected that the species detected by UV at 280 nm are mainly proteinaceous in nature, but cannot rule out the additional presence of aromatic compounds such as polyphenols. The AGP peak has a higher intensity than the main AG peak, since it has previously been shown to contain more protein (Randall, Phillips and Williams, 1989). The very small shoulder observed at elution volumes  $\sim 10.0 - 11.0$  mL is attributed to the glycoprotein (GP) fraction previously identified (Randall, Phillips and Williams, 1989). It is of interest to note that, there is also a peak corresponding to  $V_t$  which may be due to single amino acid or phenolic molecules present in the samples. Interestingly, there are also a number of peaks observed at elution volumes greater than  $V_t$ , indicating that these components must interact with the column matrix. The LS profiles clearly demonstrate the presence of two molecular mass

species, eluting at 8.0 mL and 9.0 mL, corresponding to the AGP and AG fractions respectively. It is noted that there is no significant difference observed between the molecular mass characteristics of the Sudanese and Nigerian samples.

The RI profile for the *A. seyal* gum sample (Figure 2.7) shows a peak maximum at ~9.0 mL, but there was little evidence of a shoulder present corresponding to the AGP fraction as observed for the *A. senegal* samples. This is consistent with previous studies (Siddig, et al., 2005; Hassan, et al., 2005), which have indicated the presence of a much smaller AGP content for the gum obtained from *A. seyal* and a lower protein content than for gum Arabic harvested from *A. senegal* (Table 2.3.).

The RI profile for this sample also shows a peak at ~12 mL in addition to the salt peak at  $V_t$  and the UV profile indicates that both of the peaks contain UV absorbing material. It is also apparent that some UV absorbing species elute after  $V_t$  as for the *A. senegal* gum samples.

The RI and Mw elution profiles for the *Acacia* gum samples are presented in Figures 2.8 to 2.11 and show that, the Mw for the AGP fraction eluting at the peak maximum (8.0 mL) for each of the samples is ~2000 kDa, while that for the AG fraction eluting at the peak maximum (9.4 mL) is ~250 kDa (Figures 2.8, 2.9 and 2.10). For the *A. seyal* sample (Figure 2.11) there is no observed peak for the AGP fraction, but the Mw for the AG fraction at the peak

maximum (9.0 mL) is ~600 kDa. These results are consistent with previously published data (Siddig, et al., 2005; Idris, Williams and Phillips, 1998; Renard, et al., 2012).

The RI and  $R_g$  elution profiles for the *Acacia* gum samples are presented in Figure 2.12 to 2.15 and showed that the *A. senegal* Sudan and NG<sub>1</sub> samples (Figures 2.12 and 2.13)  $R_g$  for the AGP component at the peak maximum (8.5 mL) is ~25 nm. This is consistent with the observations of Renard et al., (2012). As earlier stated, it is not possible to determine the  $R_g$  at the peak maximum for the AG fraction since the values are too small, i.e. <10 nm which is beyond the limits of the technique used. The  $R_g$  profile for the *A. senegal*-NG<sub>2</sub> sample is given in Figure 2.14 and shows an unexpected distortion in the line at an elution volume of ~8.0 mL. Interestingly, the  $R_g$  profile for the *A. seyal* sample also shows a distortion in the line. This observation cannot be explained at this point, other than to say that the distortion occurs at elution volumes corresponding to the AGP fraction in the case of the NG<sub>2</sub> sample indicating a different molecular structure. As discussed above, Renard et al., (2012) have shown that the AGP component can exist in two different structural forms.

$R_g$  is related to the molecular mass by the following equation.

$$R_g = K M^v \dots \dots \dots \text{Equation (1)}$$

Where, K is a constant, M is the molar mass, and the exponent,  $v$ , has a value that is dependent on the shape of the molecules.  $v$ , can

be determined from the slope of the line of the plot of  $\log R_g - v - \log M_w$  and has typical values of 0.60, 0.33 and 1.0 for random coils, homogeneous spheres and rod-like molecules respectively (Burchard, 1999). The  $\log R_g - v - \log M_w$  plots for the gum samples are presented in Figures 2.16 to 2.19. Figures 2.16 and 2.17 for the *A. senegal*-Sudan and NG<sub>1</sub> samples are linear and give values for  $v$  of 0.43 and 0.48 respectively. These values are similar to data reported previously (Williams and Langdon, 1995) and are consistent with a highly branched compact molecular structure as reported by Sanchez, et al., 2008 and Renard, et al., 2012. The plot for the *A. senegal*-NG<sub>2</sub> gum sample gave a significantly higher value for  $v$  of 0.67 indicating some structural differences (Figure 2.18). The RI elution profiles (Figures 2.8 to 2.9) indicated that these samples contain a slightly higher AGP component than the other two *Acacia* samples.

#### **2.4.2. Sugar, protein and amino acid content**

From the monosaccharide sugar composition and protein content of the gum samples are given in Table 2.2 and their corresponding amino acid compositions are presented in Table 2.3, it is noted that the *A. seyal* gum has significantly lower rhamnose and glucuronic acid content, but a higher arabinose content than the gums obtained from *A. senegal*, as has been reported previously (Anderson, et al., 1990; Williams and Phillips, 2009). Since the

core of the AG macromolecules present in gum Arabic (which constitute ~90% of the gum by weight) is believed to consist of a backbone of  $\beta$  1,3-linked galactose residues, the increased arabinose content recorded for *A. seyal* gum sample suggests that it possesses more and/or longer branches, which may account for its more compact structure. (Al-Assaf, Phillips and Williams, 2005a, Al-Assaf, Phillips and Williams, 2005b; Siddig, et al., 2005; Hassan, et al., 2005).

There are no significant differences observed between the sugar compositions of the *A. senegal* gums from Nigeria and Sudan. It is evident, however, that the protein content of the Nigerian gums is significantly higher than observed for the sample originating from Sudan. In addition, since the gum is known to be secreted within a zone between the inner bark and the cambial region as part of the plants wound/stress response, Joseleau and Ullman, (1990), the observed difference in protein content could be due to genotypic differences between the Nigerian and Sudanese trees from which the gum was harvested, the age of the trees, prevailing climatic conditions, the soil in which the trees are planted, attack by herbivores, plant pathogens or a combination of factors (Idris, Williams and Phillips, 1998; Siddig, et al., 2005; Al-Assaf, Phillips and Williams, 2005a; Renard, et al., 2006). Furthermore, the Nigerian samples were harvested from a small number of trees, of known genotype, grown in two different eclocations



In relation to the application of the gum in the Food Industry, it has been shown previously that the ability of *Acacia* gum exudates to stabilise oil-in-water emulsions is due to the proteinaceous components present within the gum (Padala, Williams and Phillips, 2009). Hence, the Nigerian samples may have enhanced emulsification properties. The *A. senegal*-NG<sub>1</sub> gum samples were shown to contain approximately twice the amount of protein as compared to the gum obtained from *A. seyal*, which in agreement with previous findings (Idris and Haddad, 2012). The gum obtained from *A. seyal* is not able to effectively stabilise oil-in-water emulsions. The principal amino acids present in all the gum samples are hydroxyproline, serine, aspartame, threonine and proline which are present in a ratio which approximates to 4:2:1:1:1, which is in agreement with literature values (Anderson, et al., 1990). The difference observed between the three *A. senegal* samples is that a significantly higher value for phenylalanine (0.130 g/100 g) was observed for the *A. senegal*-NG<sub>2</sub> sample compared to the *A. senegal*-NG<sub>1</sub> (0.016 g/100 g) and the *A. senegal*-Sudan gum sample (0.0745 g/100 g).

#### **2.4.3. Molecular association and hydrodynamic radius**

The molecular association of the *Acacia* gum samples was followed by monitoring the hydrodynamic size as a function of time using dynamic light scattering. The natural log of the ratio of the initial

hydrodynamic diameter to the diameter at time,  $t$ , ( $\ln d/d_0$ ) is given as a function of time in Figures 2.21 and 2.22. Figure 2.21 presents the results obtained in deionised water and shows that  $\ln d/d_0$  increases over time for the three *A. senegal* gum samples in the order  $NG_1 > NG_2 > \text{Sudan}$  indicating that molecular association occurs. It is noted that there is an almost negligible increase for the *A. seyal* sample. The results presented in Figure 2.22 in the presence of 0.5 M  $\text{NaNO}_3$ , show that  $\ln d/d_0$  remains constant over time for all of the samples indicating that molecular association does not occur. Li et al., (2009) also determined the hydrodynamic size of *A. senegal* gum samples by dynamic light scattering in both water and 6 M urea. Samples were dissolved overnight and they found that the hydrodynamic size increased with increasing gum concentration and became particularly significant above gum concentrations of  $\sim 3\%$ . The increase in hydrodynamic size was more pronounced in water compared to 6 M urea and was reduced in both solvents after filtration. The increase in size was attributed to molecular association and since the size was lower in the presence of 6 M urea compared to water it concluded that association was due to hydrogen bonding. In the current study, it is interesting to note that the increase in hydrodynamic size for the samples follows the same trend as the sample protein content i.e.  $NG_1 > NG_2 > \text{Sudan} > \text{Seyal}$ . It is belief that the molecular association results from electrostatic interaction between glucuronic acid and

protein residues on different molecules, thus forming electrostatic complexes. The fact that the hydrodynamic size does not change in the presence of 0.5 M NaNO<sub>3</sub>, supports this observation since the electrolyte would screen electrostatic interactions. This concept also supports the previous hypothesis that gum Arabic forms multilayers at the oil-water interface due to protein-glucuronic acid electrostatic interaction (Padala, Williams, and Phillips 2009; Williams, 2012).

The z-average hydrodynamic radius,  $R_h$ , of the gum samples was determined in the presence of 0.5 M NaNO<sub>3</sub> as a function of their concentration and the results are presented in Figure 2.23.  $R_h$  was obtained by extrapolation to zero concentration and the results are presented in Table 2.1, the ratio of  $R_g/R_h$  also has characteristic values depending on the shape of the molecules with values of 0.778 for a homogeneous sphere and 1.78 for a random coil (Burchard, 1999). The values obtained for the *Acacia* gum samples of 1.0 - 1.4 are similar to those reported previously and are consistent with a highly branched compact structure (Idris, Williams, and Phillips, 1998).

## **Summary**

This study represents the first detailed comparison of Nigerian and Sudanese gum Arabic samples harvested from both *A. senegal* and *A. seyal*. The results showed that the *A. seyal* gum had a lower

ramnose and glucuronic acid content than the *A. senegal* samples, but had a higher arabinose content. The sugar composition of the *A. senegal* gum samples obtained from Nigeria were generally similar to that obtained from Sudan although slight variation was observed. However, the protein content of the Nigerian gums was shown to be significantly higher than that of the sample originating from Sudan. In addition, all *A. senegal* gum samples were shown to contain at least twice the amount of protein as compared to the gum obtained from *A. seyal*.

Gel permeation chromatography of the samples coupled to light scattering, refractive index and UV detectors, showed the presence of arabinogalactan, arabinogalactan protein and glycoprotein fractions and also shown the presence of an additional small proportion of very low molar mass proteinaceous material in all the samples which has previously been ignored. The plot of radius of gyration,  $R_g$ , showed a discontinuity for one of the Nigerian samples (*A. senegal*-NG<sub>2</sub>) and for the *A. seyal* gum sample suggesting a different molecular structure. Plots of  $M_w$  versus  $R_g$  confirmed that the molecules had a compact structure.

### **3.0. Chapter Three**

#### **3.1.0. An investigation of the emulsification properties of gum Arabic**

##### **3.1.1 Introduction**

In the food industry, emulsions are prepared using equipment that produces high shear forces, such as high pressure homogenizers that emulsify oil and an aqueous phase together, in the presence of a surface active agent such as gum Arabic (McClements, 2005). This process involves forcing a coarse mixture of oil and aqueous phase together through a very narrow aperture or slit under high pressure, resulting in an intense laminar shear flow, cavitation and turbulence while the surface active agent (emulsifier) is adsorbed on to the oil-water interface. This process thereby creates a stabilizing, interfacial layer at the oil droplet surface, leading to the development of fine, uniformly dispersed droplets (Dickinson, 2003; Dickinson, Radford and Golding, 2003).

There are three main types of emulsion used in the food industry: Oil-in-Water (O/W) emulsions, Water-in-Oil (W/O) emulsion and multiple emulsions made up of Oil-in-Water-in-Oil (O/W/O) (Kim, Decker and McClements, 2006). The work presented in this chapter is mainly concerned with oil-in-water (O/W) emulsions.

Oil-in-water emulsions consist of small oil droplets dispersed in an aqueous medium, with each droplet being coated with a thin layer of an emulsifier (McClements, 2012). In such oil-in-water

emulsions, droplets of oil are suspended in a continuous aqueous phase (Donsi, Wang and Huang, 2011; Donsi, Sessa and Ferrari, 2012).

In the literature, the terms *emulsifier* and *stabilizer* are often used interchangeably. However, although most hydrocolloids can act as stabilizers (stabilizing agents) of O/W emulsions, only a few can act as emulsifiers. In fact, an emulsifier can be defined as a single chemical species or mixture of species that supports emulsion formation and stabilization by interfacial action (Dickinson, 2003). A stabilizer is a single chemical species, which can give long-term stability to an already formed emulsion, (it may not necessarily be an emulsifier) possibly by a mechanism of adsorption (Garti and Leser, 2001). Emulsifiers (emulsifying agents) are substances with substantial surface activity at the oil-water interface, and have the ability to expedite the formation and stabilization of fine droplets during and after emulsification (Dickinson, 2003; 2004).

Food grade emulsifiers such as gum Arabic often contain macromolecular proteins (amphiphilic high molecular weight molecules) which in aqueous solution, have a tendency to fold in a coil-like structure so as to uncover the hydrophilic groups to the water and cover the hydrophobic segments in the centre of the coil. However, when the protein molecule reaches an oil-water interface, the molecule will partially unfold and orient its hydrophobic groups towards the oil phase (Pagnaloni, et al., 2004).

Gum Arabic has been reported to possess both foaming and interfacial activity which enables it to migrate slowly to air-water and oil-water interfaces to make stable foam and emulsions (Garti et al., 1997).

Currently, it is thought that it is the arabinogalactan-proteins and other glycoproteins present in gum Arabic that are responsible for the emulsifying ability of the gum (Randall, Phillips, and Williams, 1988; Randall, Phillips, and Williams, 1989). The proteinaceous moiety of these molecules are hypothesised to firmly anchor to the oil-water interface, while the charged polysaccharide segments protrude into aqueous phase, thereby providing a strong steric stabilization for the emulsion. The adsorption of gum Arabic at oil-water interface was first studied in detail by Randall, Phillips, and Williams, 1988; BeMiller, 1988, and Randall, Phillips, Williams, (1989), with the view to elucidating the quantity of gum required to make a stable emulsion. Dickinson, (1989) and Lopez-Franco, et al., (2004) studied the relationship between interfacial rheology and the emulsification performance of the gum using covent-type surface rheometers at the oil-water interface, and the effect of extensive dilution of the aqueous phase, the result indicated that only a small fraction containing nitrogen adsorbed at the oil-water interface.

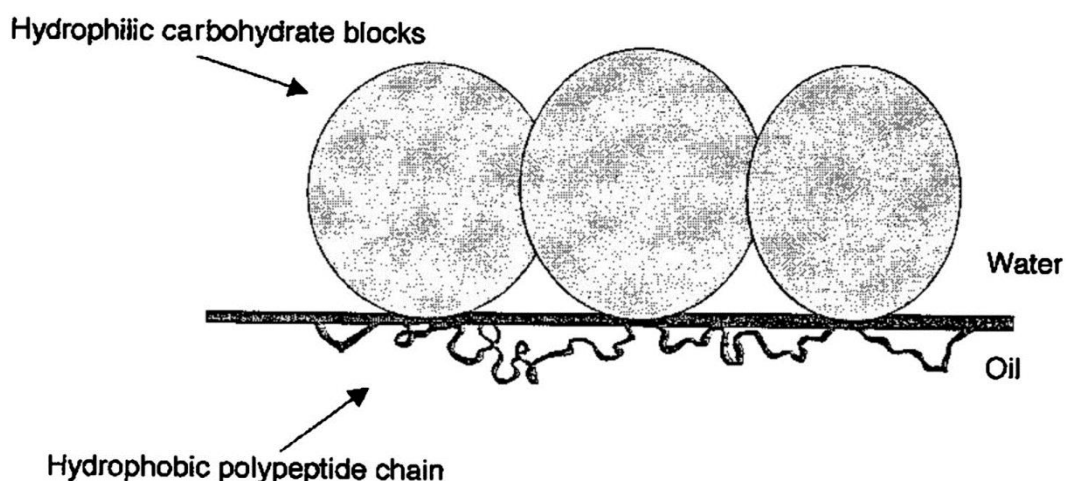
### **3.1.2. Emulsion stability**

The stability of an emulsion can be described as the ability of an emulsion to resist change to its properties over time. Emulsifiers which provide smaller emulsion droplets and a more uniform emulsion produce more stable emulsions. The appropriate use of emulsifiers and their ability to stabilize emulsions effectively, largely depend on the resultant droplet size and distribution (Fernandez, et al., 2004; Jurado et al., 2007; Achouri, Zamani and Boye, 2012).

El-Kheir, Abu El-Gasim and Baker, (2008) reported that, the stability of gum Arabic emulsions was significantly affected by the types of oil used and the source of the gum. The adsorbed gum Arabic surface layer is able to prevent droplet flocculation and coalescence through both electrostatic and steric repulsive forces (Funami et al., 2008; Li et al., 2009).

Wang, et al., (2011), studied the effects of the addition of (0 - 4% w/w) gum Arabic on stability of oil-in-water emulsion stabilized by flaxseed protein concentrate (FPC) and soybean protein concentrate (SPC). The results demonstrated that emulsions stabilized by both proteins in the presence of the 2% (w/w) gum Arabic have better stability compared to when the gum is not added.





**Figure 3.1.** Schematic representation of gum Arabic adsorbed onto oil droplets. (Adapted from Evans, Ratcliffe and Williams, 2013).

The separation of adsorbed components of oil droplets in gum Arabic stabilized emulsions using the sodium dodecyl sulphate (SDS) desorption method from 15% (w/w) oil emulsions, showed that the amount adsorbed onto the oil is only 1 - 10% of the total gum (Katayama et al., 2006). Further analysis of the adsorbed fraction of two different molecular weight gum Arabic samples using GPC-RI, revealed that the amount adsorbed in the two resultant emulsions was very different. Therefore, the authors suggested that, the molecular weight of the adsorbed component of gum Arabic on the oil surface is an important aspect for emulsion stability compared with the quantity of gum adsorption (Katayama et al., 2006).

In recent years, there has been increased interest in natural compounds that consist of protein-polysaccharide complexes (such as gum Arabic), and their applications as emulsifiers for the encapsulation of active compounds, (Tan, 2004; Sikora et al., 2008; Vinayahan, Williams and Phillips, 2012; Evans, Ratcliffe and Williams, 2013). As such, an improved understanding of the extent to which gum Arabic is adsorbed during the formation of stable emulsions is necessary for effective utilization of the product.

Gum Arabic sourced from either *A. senegal* or *A. seyal* is acceptable within the regulatory specification of gum Arabic used in the food industry (Elmanan, et al., 2008). However, that harvested from *A. senegal* is more commonly used commercially due to its better emulsification properties for application in oil-in-water emulsions, cosmetic products and inks etc (Flindt et al., 2005). Gum harvested from *A. seyal* therefore tends to be used for applications where long term emulsion stability is not required.

The objective of the study presented in this chapter, was to gain a clearer understanding of the mechanisms underlying the differences in the emulsification properties exhibited by *A. senegal* and *A. seyal* gum exudates. For this investigation, polystyrene lattices were selected as a model system to determine the adsorbed layer thickness, which was studied using a combination of dynamic light scattering and gel permeation chromatography (Robinson and Williams, 2002; Padala, Williams and Phillips,

2009). According to Hunter et al, (2008), the droplet size distribution of emulsion gives the best information on evaluation of the emulsion's properties and therefore, the droplet size of the emulsions prepared for this study were evaluated by applying the laser diffraction technique, using the Mastersizer 2000 (Malvern Instrument). The specific surface area (SSA) (i.e. the surface area of all emulsion droplets per unit volume of emulsion) is a very good indicator in the determination of the emulsifying activity of an emulsifier. The minimum emulsion droplet size and maximum surface area per unit volume of oil are required to have a stable emulsion (Dickinson, 1998; Leal-Calderon, Schmitt and Bibette, 2007; Dickinson, 2009a; Dickinson, 2009b), which was also considered in this work. In addition to which, the polystyrene lattices in the presence and absence of the two gums was observed via transmission electron microscopy (using negative staining) and the results compared to the results obtained using the biophysical analyses.

### **3.2.0. Materials and methods**

#### **3.2.1. Materials**

The samples of *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gums used in this study have been described previously in chapter 2.

Polystyrene latex particles of 0.1 µm mean particle size were purchased from Sigma-Aldrich Chemie GmbH, Germany and in the form of a 10% dispersion. The bovine serum albumin (BSA) was obtained from Sigma Aldrich, Gillingham, UK. Sodium nitrate (analytical grade) and D-Limonene reagents were obtained from Fisher Scientific, UK. The density of the D-limonene was 0.843 g/cm<sup>3</sup>.

#### **3.2.2. Methods**

##### **3.2.2.1. Molecular mass distribution**

The molecular mass distribution of the samples was determined by gel permeation chromatography (GPC). The system comprised of a Superose 6 column and a vacuum degasser (type 006150/4 Cambridge, U.K). The eluent used was 0.1 M sodium nitrate containing 0.005% sodium azide (which acts as a bactericide) filtered with a GSWP 0.22 µm Millipore membrane filter, under reduced pressure before use. The samples were passed through a 0.45 µm pore size syringe filter prior to injection into the system, through a rheodyne injection valve (Rheodyne Inc., USA) fitted with a 200 µm volume loop and delivered at constant flow rate of

0.5 mL/min using an HPLC pump (Waters corporation 515). The eluent from the column was monitored by multi-angle laser light scattering (MALLS) in conjunction with an Optilab DSP refractive index (RI) detector (Wyatt Technology) and an Agilent 1100 UV spectrophotometer detector set at a wavelength of 280 nm. The refractive index increment (dn/dc) value used was 0.141 mL/g (Padala, Williams, and Phillips, 2009). Data was captured using ASTRA 4.90.80 software (Wyatt Technology).

#### **3.2.2.2. Hydrodynamic size**

The hydrodynamic size of the *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gum samples and BSA (control) were determined by dynamic light scattering using the Zetasizer, Nano series ZS, (Malvern Instruments Ltd, UK) equipped with a 5 Mw He-Ne laser ( $\lambda_0$  632 nm).

Powdered samples were dissolved in sterile deionized water and in 0.5 M NaNO<sub>3</sub> to make up 0.05% w/w. These solutions were filtered using a 0.45  $\mu$ m pore size filter in order to remove extraneous materials immediately before transfer of 1.5 mL of each into a DTS 0012 disposable cuvette and placing them into the measuring chamber of the instrument. All measurements were performed at room temperature (25 °C) and recorded from an average of 10 sub-runs.

### **3.2.2.3. Determination of the adsorbed layer thickness**

The thickness of the gum Arabic layer adsorbed onto the polyetyrene latex particles was determined by dynamic light scattering. Gum Arabic or BSA (9.5 mL) solutions at concentrations of 0.01 - 0.05% in water and in presence of 0.5 M NaNO<sub>3</sub> were added to 0.5 mL of a 0.5% polystyrene latex particles dispersion. The hydrodynamic size of the particles with polymer adsorbed was determined and the adsorbed layer thickness was calculated from the difference in the hydrodynamic size of the particles with and without adsorbed polymer. The thickness of the adsorbed layer was determined as a function of time.

### **3.2.2.4. Preparation of emulsions**

Limonene oil-in-water emulsions were prepared according to the method described by Padala, Williams, and Phillip, (2009), in which 32 g of 0.5% (w/w) gum Arabic (*A. senegal* and *A. seyal* as emulsifiers) in water was accurately weighed followed by the addition of 8 g of D-limonene to make a total of 40 g (20% w/w). Emulsion of 20% (w/w) *A. senegal* and *A. seyal* were prepared by high shear mixer (HSM) using an Ultra Turrax T25 mixer (IKA Werke GmbH and co DE) equipped with a S25 N18 G rotor set at maximum 24,000 rpm for 4 minutes at temperature of 25 °C.

### **3.2.2.5. Amount of gum adsorbed**

The prepared emulsions were left to equilibrate for 72 hours prior to centrifugation, the supernatant was collected and the molecular mass distribution determined via GPC. The difference in the RI peak areas before and after emulsification was obtained using the Astra software 4.90.20 and the amount of gum Arabic adsorbed onto the oil droplets calculated.

### **3.2.2.6. Determination of emulsion droplet size**

The droplet size and mean specific surface area of the gum Arabic oil-in-water emulsion was determined by laser diffraction, using Mastersizer 2000 (Malvern Instruments).

Laser diffraction measures particle size distribution by measuring the angular variation in the intensity of scattered light as the laser beam passes through a dispersed particulate sample. Larger particles scatter light at small angles relative to the laser beam and smaller particles scatter light at large angles. The angular scattering intensity data is then analysed by the system to calculate the size of the particles responsible for the scattering pattern, using the Mie theory of light scattering as adopted by (Sochan, Polakowski and Lagod, 2014).

In order to perform the measurements, the dispersion unit of the instrument was first cleaned with distilled water while simultaneously varying the agitation speed until the laser intensity

display was about 80%. The emulsion was then added to the water in the dispersion unit dropwise using a pipette, until the obscuration was between 10 - 15%. All measurements were performed in triplicate, at room temperature.

### **3.2.2.7. Transmission electron microscopy**

The adsorption of the two gum Arabic samples (*A. senegal*-NG<sub>1</sub> and *A. seyal*) onto polystyrene latex particles was investigated by transmission electron microscopy. A 9.5 mL of the *A. senegal*-NG<sub>1</sub> and *A. seyal* solution at a concentration of 1% (w/w) in sterile deionised water was added to 0.5 mL of a 0.5% polystyrene latex particles dispersion.

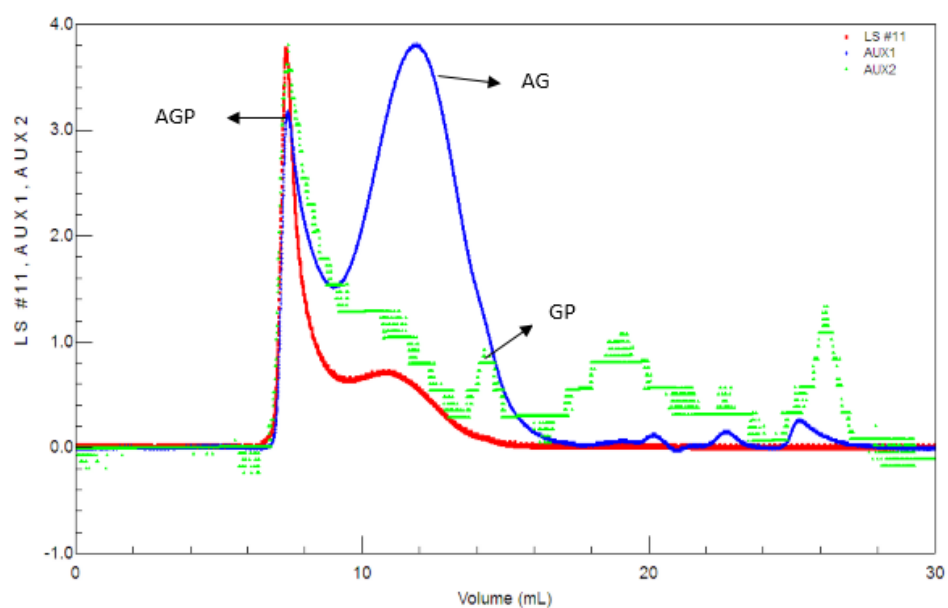
The grids were negatively stained by floating on a 30 µl drop of 2% (w/w) uranyl acetate for 90 seconds. Excess uranyl acetate was removed by carefully touching the edge of the grids with a piece of filter paper (wicked). The negatively stained grids were then air dried, prior to being observed on a JEOL JEM-1200 EX Transmission Electron Microscope, at an accelerating voltage of 80 kV. The images were taken using a GATAN retractable multi scan camera.



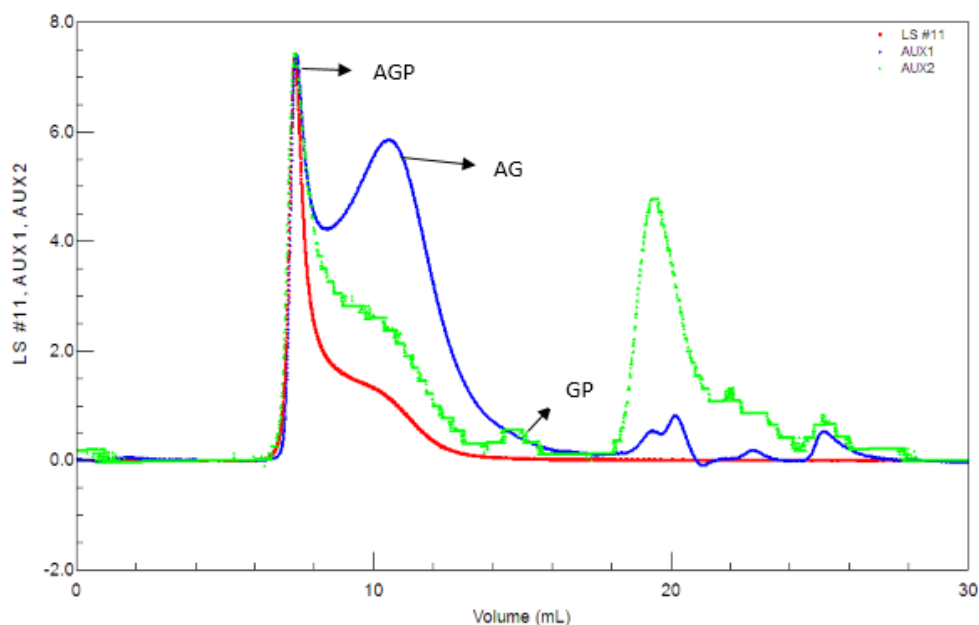
### **3.3.0. Results**

#### **3.3.1. Physicochemical characterisation**

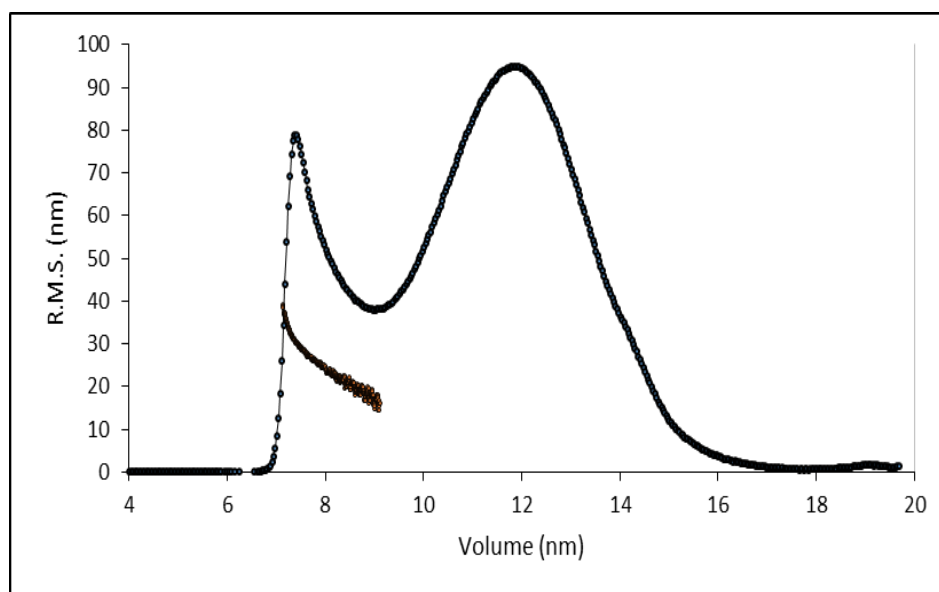
The GPC elution profiles showing the major components of the gum Arabic samples using light scattering, refractive index and U.V (at 280 nm) detectors are presented in Figures 3.2 and 3.3 for *A. senegal* and *A. seyal* respectively, while Figures 3.4 and 3.5 show the *A. senegal*-NG<sub>1</sub> and *A. seyal* RI and radius of gyration profiles. The separation of molecules was performed using a Superose 6 column with sample delivered at constant flow rate of 0.5 mL/min.



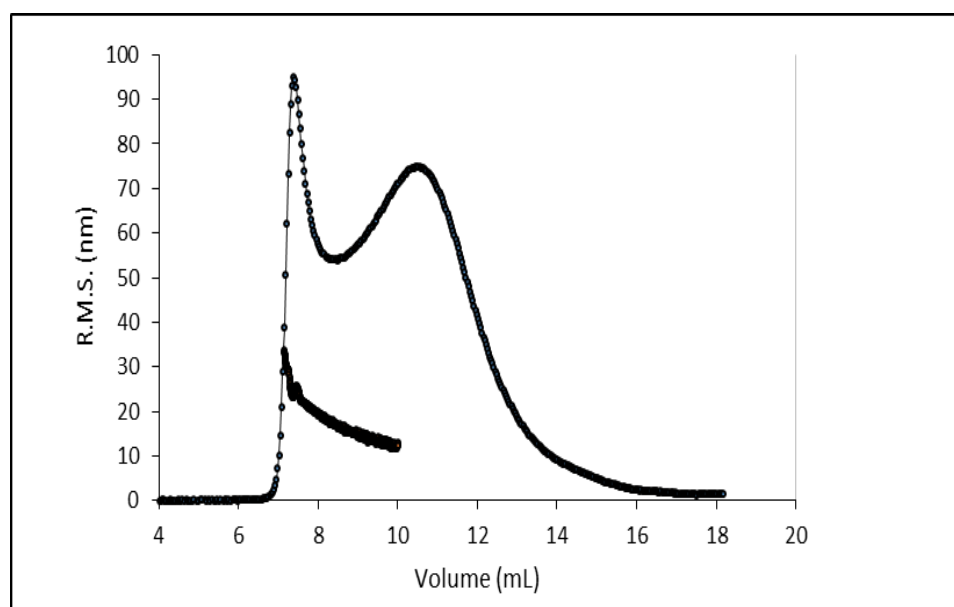
**Figure 3.2.** GPC elution profile of gum Arabic (*Acacia senegal*-NG<sub>1</sub>) monitored using MALLS, RI and UV detector at 280 nm. (where, AGP= Arabinogalactan Protein, AG= Arabinogalactan, and GP= Glycoprotein). This is applicable to Figure 3.3 below for *A. seyal*.



**Figure 3.3.** GPC elution profile of gum Arabic (*Acacia seyal*) monitored using MALLS, RI and UV detector at 280nm.



**Figure 3.4.** RI and radius of gyration (R.M.S) elution profile of gum Arabic (*Acacia senegal*-NG<sub>1</sub>) gum sample. (R.M.S. = Root Mean Square radius of giration).



**Figure 3.5.** RI and radius of gyration (R.M.S) elution profile of gum Arabic (*Acacia seyal*) gum sample.

### 3.3.2. Hydrodynamic sizes

The hydrodynamic sizes of the different materials used in this study are presented in Table 3.1. These values were used for the determination of the adsorbed layer thickness of the gum Arabic samples.

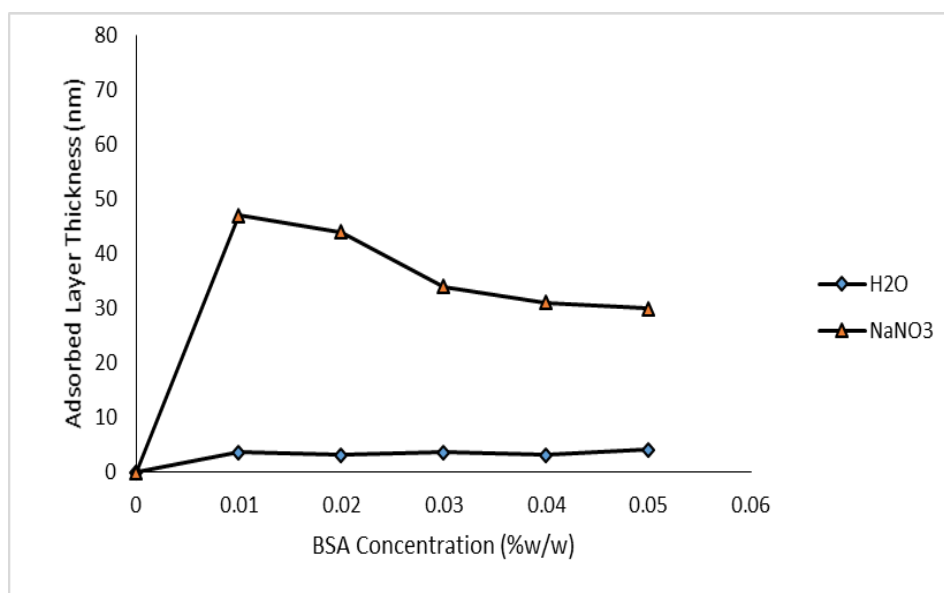
**Table 3.1.** Hydrodynamic size (Z-Average diameter and radius) of different materials involved in the determination of adsorbed layer thickness of the gum Arabic samples (*Acacia senegal* and *Acacia seyal*).

	Z-Average Diameter	Z-Average Radius
0.05% <i>A. senegal</i> - NG <sub>1</sub> in water	29.8	14.9
0.05% <i>A. seyal</i> in water	34.2	17.1
0.5% BSA in water	6.11	3.1
0.5% Polystyrene latex particles	98.7	49.4

The 0.05% concentration used for the gum samples is the highest in the range of concentrations (0.01 - 0.05%) used in the analyses.

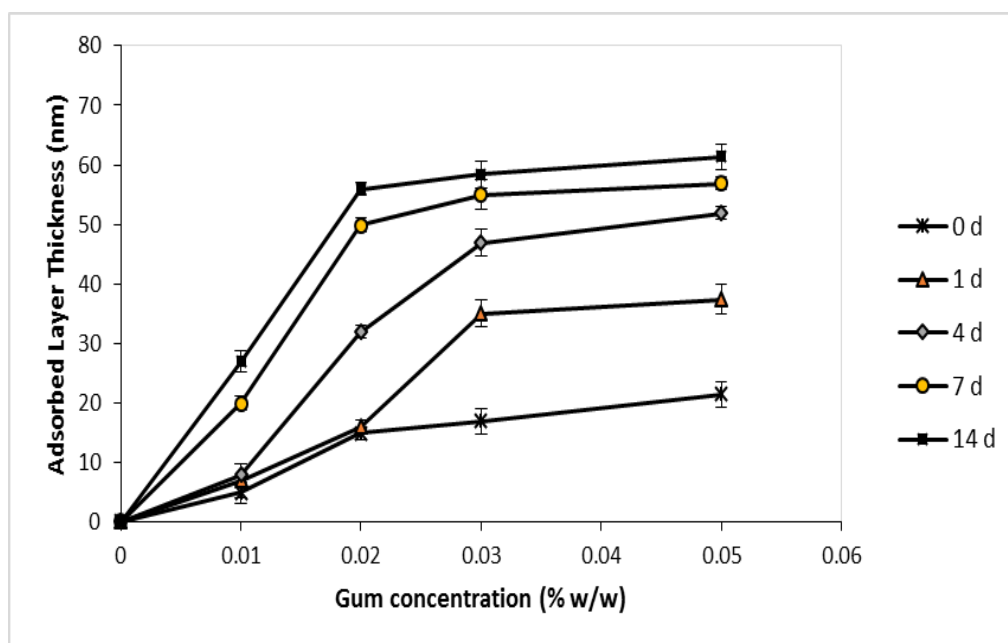
### 3.3.3. Adsorbed layer thickness

The adsorbed layer thickness of BSA adsorbed onto polystyrene latex particles in the presence of varying concentrations of BSA in water and in 0.5 M NaNO<sub>3</sub> is presented in Figure 3.6. The adsorbed layer thickness in water at varying concentration of the BSA were seen to be very small. However, a sharp increase was observed in 0.5 M NaNO<sub>3</sub> which later declined.

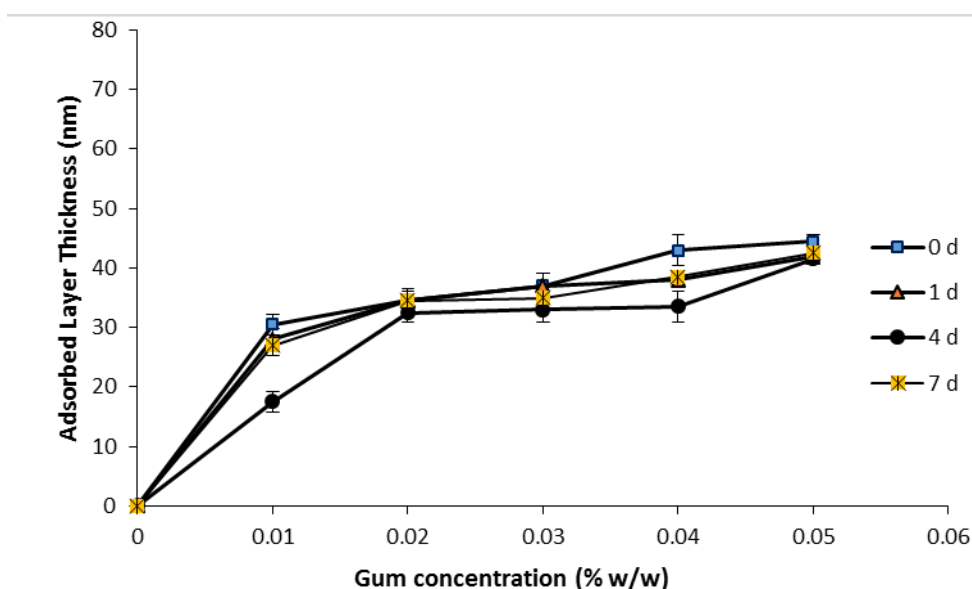


**Figure 3.6.** Adsorbed layer thickness of BSA as a function of concentration in water and in 0.5 M NaNO<sub>3</sub>. This was done to test the robustness of the experimental protocol and was carried out only once without replication.

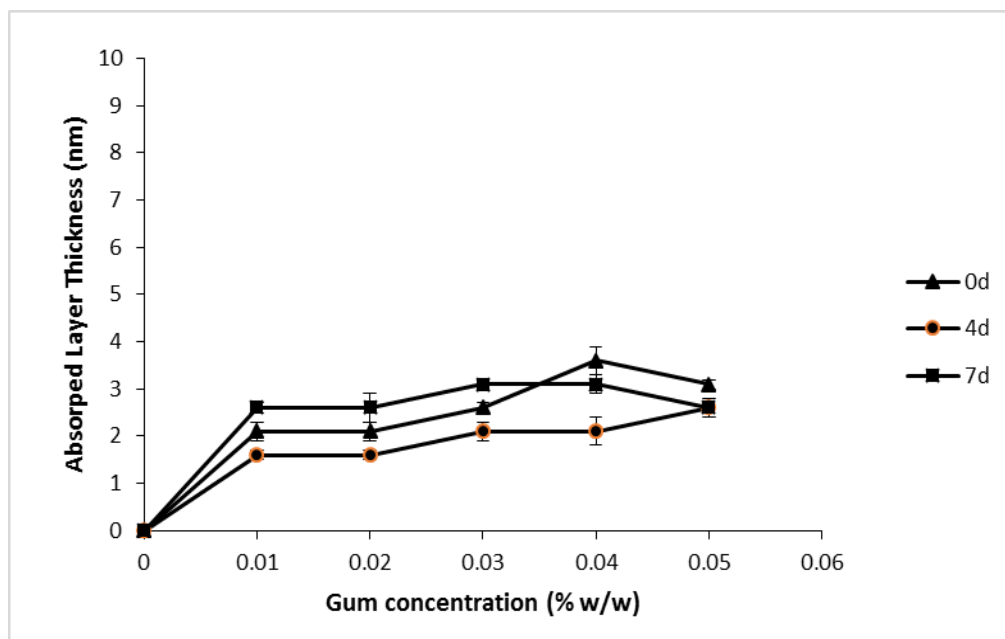
The adsorbed layer thickness of the *Acacia* gum samples (both *A. senegal*-NG<sub>1</sub> and *A. seyal*) are presented in Figures 3.7 to 3.10. Figures 3.7 and 3.8 show the adsorbed layer thickness for *Acacia senegal*-NG<sub>1</sub> gum in water and in salt solution (0.5 M NaNO<sub>3</sub>). A significant increase was observed in water over a period of 14 days, while little change was observed in the presence of salt. The adsorbed layer thickness for *A. seyal* gum is presented in Figure 3.9 and 3.10 and show that the values of the increment are very much less compared to the *A. senegal*-NG<sub>1</sub> gum sample.



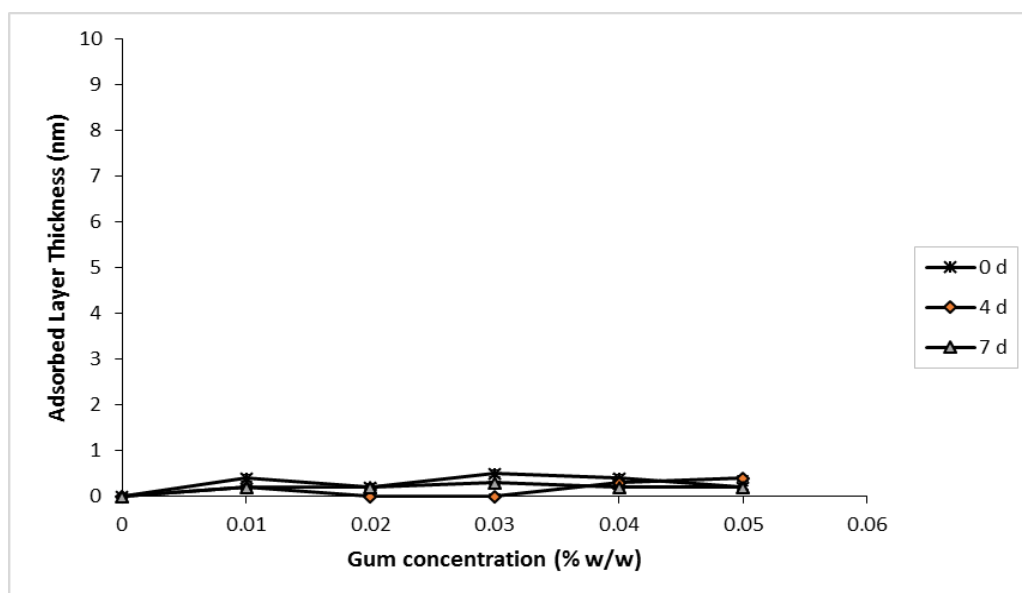
**Figure 3.7.** Adsorbed layer thickness of *Acacia senegal*-NG<sub>1</sub> gum sample adsorbed onto polystyrene latex particles as a function of concentration in water over 14 days period. (Where, d= day). The result is a mean of three replcations.



**Figure 3.8.** Adsorbed layer thickness of *Acacia senegal*-NG<sub>1</sub> gum sample adsorbed onto polystyrene latex particles as a function of concentration in 0.5 M NaNO<sub>3</sub> over a 7 day period. (Where, d= day). The result is a mean of three replcations.



**Figure 3.9.** Adsorbed layer thickness of *Acacia seyal* gum sample adsorbed onto polystyrene latex particles as a function of concentration in water over a 7 day period. (Where d=day). The result is a mean of three replcations.

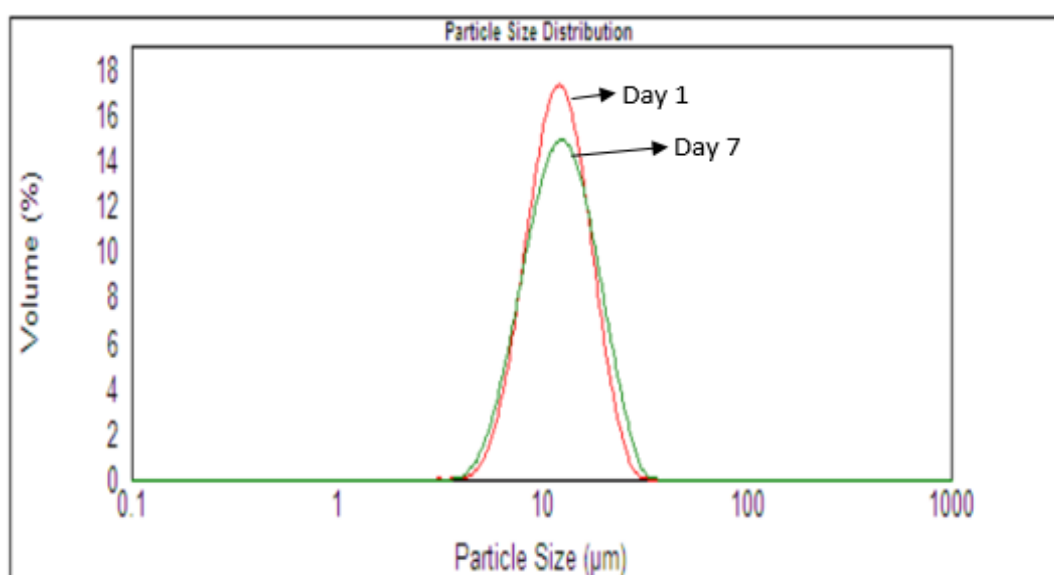


**Figure 3.10.** Adsorbed layer thickness of *Acacia seyal* gum sample adsorbed onto polystyrene latex particles as a function of concentration in 0.5 M  $\text{NaNO}_3$  over 7 days period. (Where d=day). The result is a mean of three replcations.

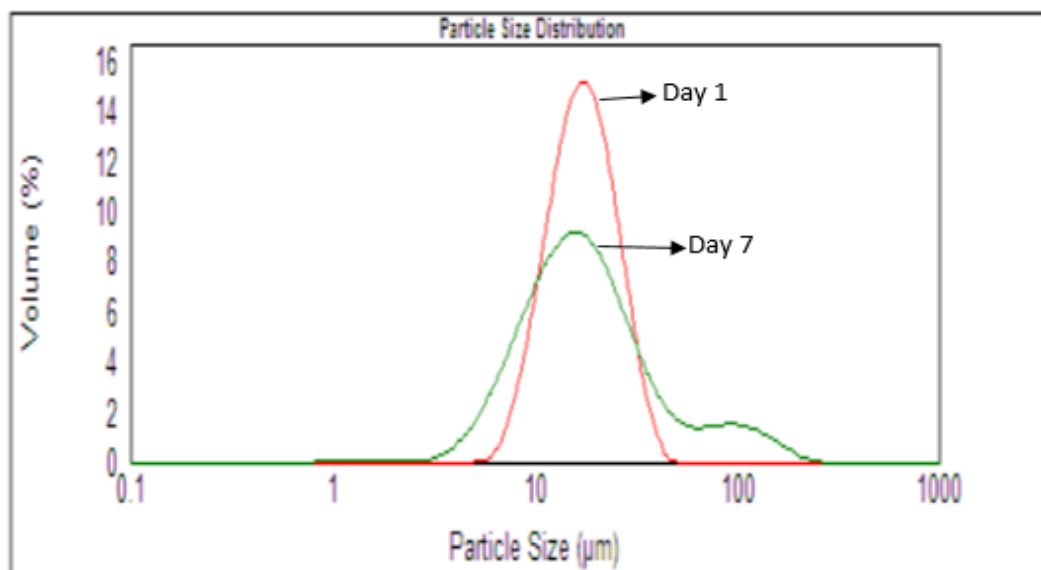


### 3.3.4. Droplet size distribution

The droplet size distribution for the freshly prepared limonene oil-in-water emulsions and the emulsions stored for 7 days for both *A. senegal*-NG<sub>1</sub> and *A. seyal* are presented in Figures 3.11 and 3.12 below. It can be clearly observed from these results that the droplet size for the emulsion prepared using *Acacia seyal* increases significantly, whilst that of the emulsion prepared using *Acacia senegal* remains the same even after a 1 week in storage.



**Figure 3.11.** Droplet size distribution of limonene oil-in-water emulsion prepared with gum Arabic (*Acacia senegal*-NG<sub>1</sub>) stored over 7 day period.



**Figure 3.12.** Droplet size distribution of limonene oil-in-water emulsion prepared with gum Arabic (*Acacia seyal*) stored over 7 day period.

Tables 3.2 and 3.3 gives the mean surface area parameters for *A. senegal* and *A. seyal* respectively, used in determining the stability of the emulsion prepared with the gums. These parameters are the Volume mean diameter  $D[4,3]$ , Surface mean diameter  $D[3,2]$  and the specific surface area (SSA). The values for *A. senegal*-NG<sub>1</sub> are less than that of *A. seyal*, indicating that *A. senegal* is more stable and gives a better emulsifier.

**Table 3.2.** Droplet size for emulsion prepared using gum Arabic (*Acacia senegal*-NG<sub>1</sub> (Limonene oil-in-water emulsion)).

Day	D[3,2]	D[4,3]	SSA (m <sup>2</sup> /g)
0	11.4	12.7	0.5
7	11.5	13.2	0.5

Where, SSA = specific surface area. D[3,2] = surface mean diameter and D[4,3] = volume mean diameter. All values are mean of three readings.

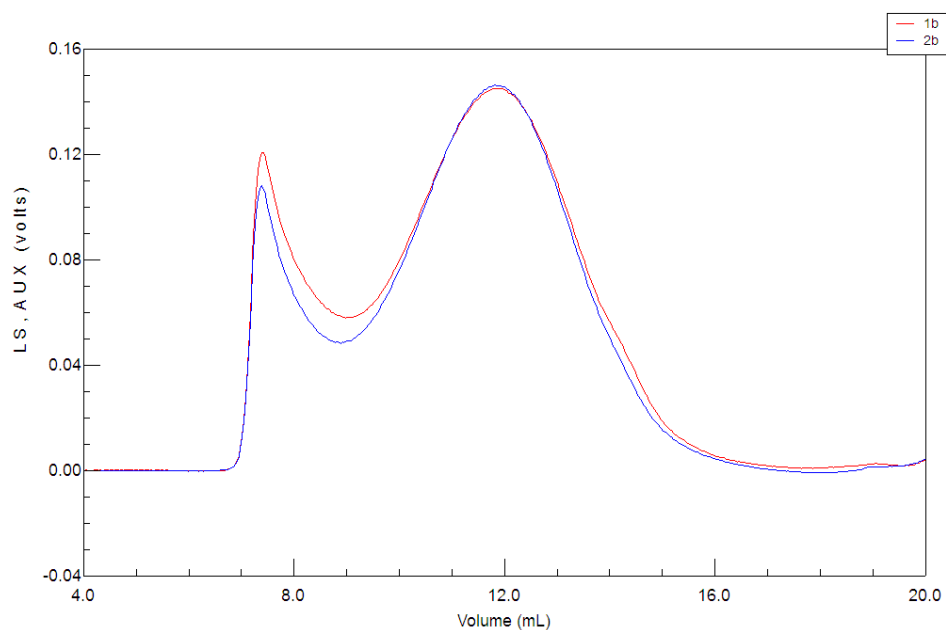
**Table 3.3.** Droplet size for emulsion prepared using gum Arabic (*Acacia seyal*) (Limonene oil-in-water emulsion)

Day	D[3,2]	D[4,3]	SSA (m <sup>2</sup> /g)
0	15.9	18.3	0.3
7	14.0	26.8	0.4

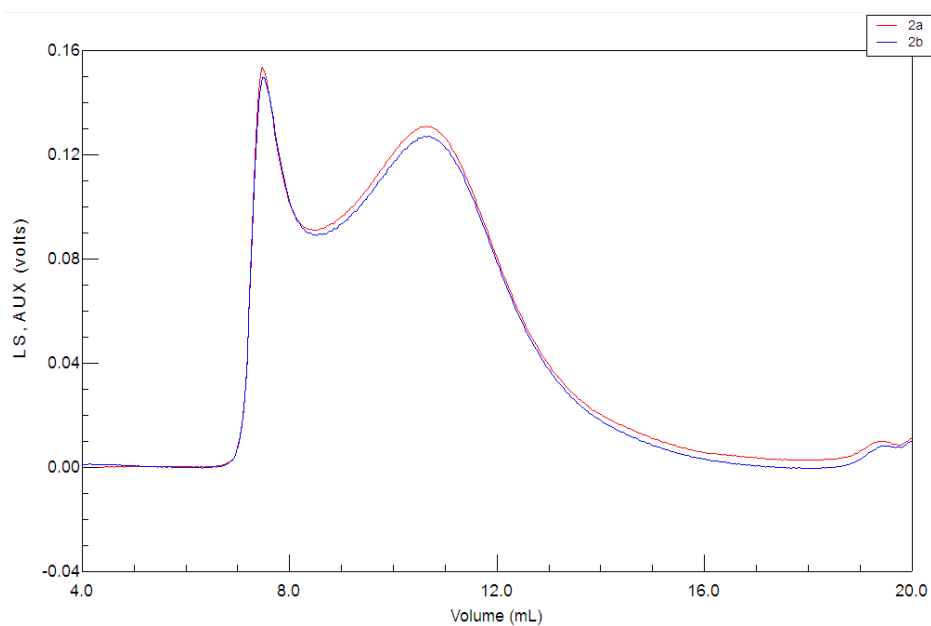
Where, SSA = specific surface area. D[3,2] = surface mean diameter and D[4,3] = volume mean diameter. All values are mean of three readings.

### **3.3.5. Amount of gum Adsorbed**

The RI elution profiles for the gum samples before and after emulsification was monitored and the results are presented below. Figure 3.13 is the RI elution profile for *A. senegal*-NG<sub>1</sub> gum and Figure 3.14 is for the *A. seyal* gum. The upper red chromatograms are for the whole gum before emulsification while the lower blue are for the supernatants collected after emulsification and left for 1 week. The difference in the area between the two profiles gives the amount of gum adsorbed.



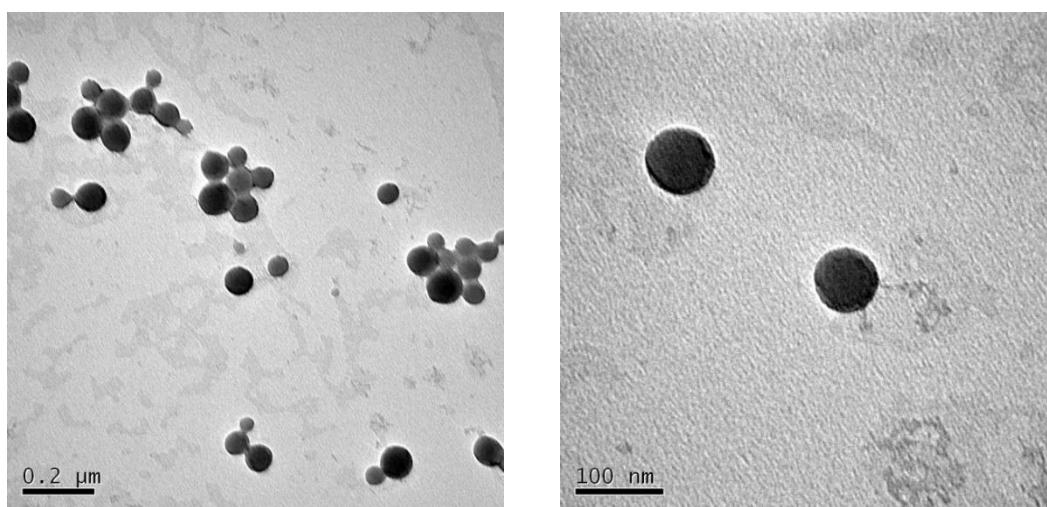
**Figure 3.13.** GPC elution profile for gum Arabic (*Acacia senegal*-NG<sub>1</sub>) monitored by RI showing profile for the emulsion (1b) and supernatant (2b) recovered from emulsion.



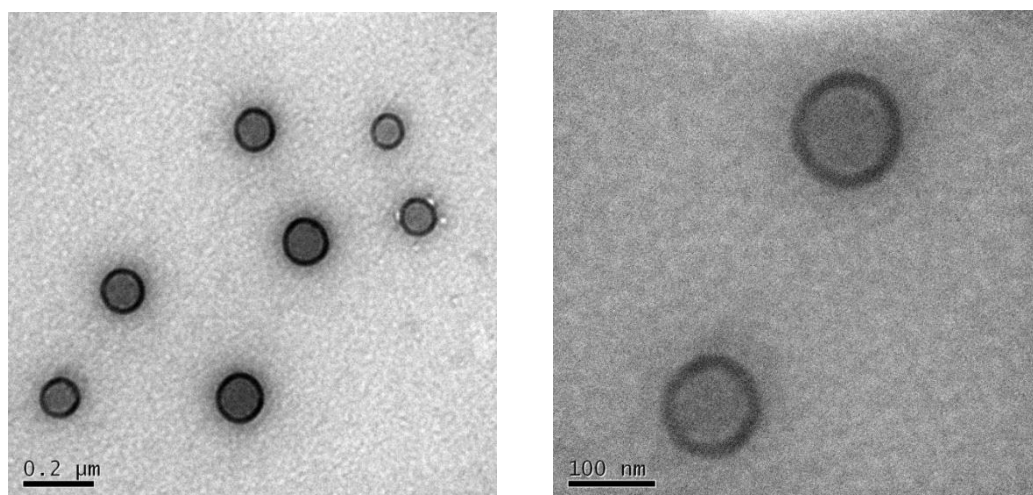
**Figure 3.14.** GPC elution profile for gum Arabic (*Acacia seyal*) monitored by RI, showing profile for the emulsion (2a) and the supernatant (2b) recovered from emulsion.

### 3.3.6. Transmission Electron Microscopy

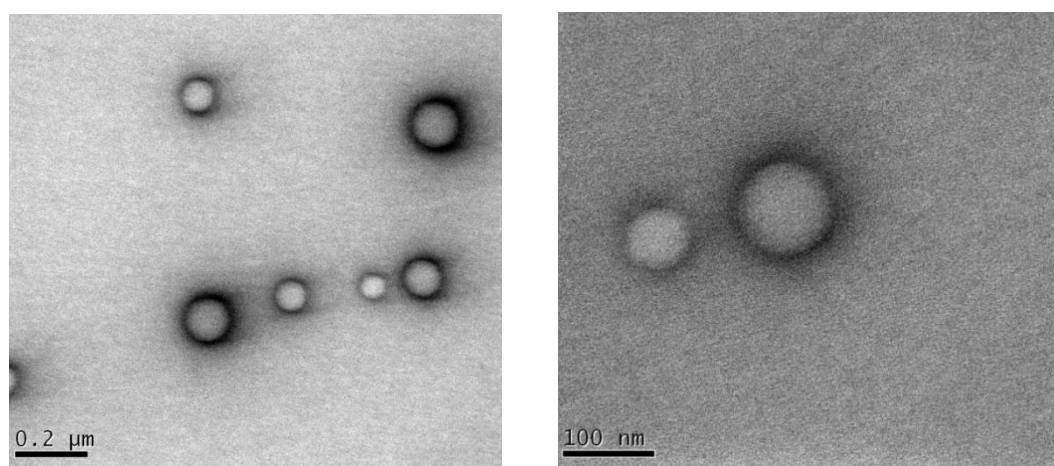
The transmission electron micrographs of the control polystyrene latex particles (not in the presence of the gum samples) are presented in Figure 3.15 and the images of those prepared in the presence of *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gums are presented in Figures 3.16 and 3.17. From these images it can be observed that there is a distinct, thick layer of gum adsorbed onto the polystyrene latex particles prepared in the presence of *Acacia senegal* gum and that only a diffuse, thinner layer is apparent on the surface of the particles prepared in the presence of the gum harvested from *Acacia seyal* (Figure 3.17).



**Figure 3.15.** Negatively stained transmission electron micrographs of the control polystyrene latex particles at X100K and X250K magnification.



**Figure 3.16.** Transmission electron micrographs of negatively stained *Acacia senegal*-NG<sub>1</sub> gum adsorbed onto polystyrene latex particles at two magnifications (X100K and X250K).



**Figure 3.17.** Transmission electron micrographs of negatively stained *Acacia seyal* gum adsorbed onto polystyrene latex particles at two magnifications (X100K and X250K).

### **3.4.0. Discussion**

#### **3.4.1. Physicochemical characterisation**

The GPC elution profiles for the *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gum samples as monitored by light scattering, refractive index and UV at 280 nm wave length are presented in Figures 3.2 and 3.3. These show the presence of the arabinogalactan protein, arabinogalactan and glycoprotein fractions as has been discussed previously in Chapter 2. The RI elution profiles of the two samples together with the radii of gyration,  $R_g$ , of the eluting species are presented in Figures 3.4 and 3.5 and are consistent with the literature, as discussed previously in Chapter 2. It is not possible to determine the  $R_g$  across the whole molecular mass profile since the samples contain molecules with  $R_g$  less than  $\sim 10$  nm which is below the limits of this technique. The z-average  $R_h$  values for the *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* were found to be 14.9 nm and 17.1 nm respectively as presented in Table 3.1.

#### **3.4.2. Hydrodynamic size**

The hydrodynamic radius of the different materials used in this study were determined and are presented in Table 3.1. It was observed that the value of the hydrodynamic radius of *A. seyal* (17.1 nm) is higher than that of *A. senegal*-NG<sub>1</sub> (14.9 nm) at the same concentration of sample and was consistent with the values in the literature (Siddig, et al., 2005; Al-Assaf and Phillips, 2006).



Similar trend was observed for the radius of gyration of the two gums with *A. senegal*-NG<sub>1</sub> and *A. seyal* having 13.0 nm and 24 nm respectively.

### **3.4.3. Adsorbed layer characteristics**

Initial studies were undertaken to determine the adsorbed layer thickness of BSA adsorbed onto polystyrene latex particles in order to test the robustness of the experimental protocol. The results are presented in Figure 3.6. The adsorbed layer thickness values obtained in water were found to be ~3 nm and are similar to values reported by others (Robinson and Williams, 2002). This value is consistent with the fact that the molecules were found to have a z-average diameter of 6.6 nm by dynamic light scattering. It is recognised that the adsorption of globular proteins onto hydrophobic surfaces is facilitated through molecular unfolding and the exposure of the hydrophobic groups within the protein core. The adsorbed layer thickness values obtained in the presence of 0.5 M NaNO<sub>3</sub> are much higher than can be expected and indicate that bridging flocculation has occurred. It is evident that the protein adsorbed layer thickness is too small to facilitate stabilisation through steric repulsive forces.

The adsorbed layer thickness values of *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gums adsorbed onto polystyrene latex particles in water and in the presence of 0.5 M NaNO<sub>3</sub> is shown as a function of

the concentration of gum added and after various equilibration times in Figures 3.7 - 3.10. Figure 3.7 shows the adsorbed layer thickness for *Acacia senegal*-NG<sub>1</sub> gum adsorbed in water. The first point to note is that the thickness increases as the amount of gum present increases and then tends to plateau. The increase is due to an increase in the amount of gum adsorbed at the surface as the gum concentration is increased. The value obtained for the adsorbed layer thickness in the presence of 0.05% gum (at plateau coverage) on Day 0 is 21 nm which compares to a value of 29.8 nm for the z-average hydrodynamic diameter of the gum molecules determined by dynamic light scattering. Since the gum Arabic molecules are believed to have a disk or cylinder-like structure with a thickness of ~5 nm (Renard, et al., 2012; Renard, et al., 2014) or less, it suggests that the molecules do not lie flat on the surface, but must adsorb end-on or else adsorb as multilayers. It is particularly interesting to note that the adsorbed layer thickness also increases significantly with time. For example, for the latex particles in the presence of 0.05% gum solution the thickness increases from 21 nm to 61 nm over a 14 day period. The most likely reason for the increase in the adsorbed layer thickness is due to multilayer adsorption which occurs as a consequence of protein amine groups and the carbohydrate carboxylate groups within molecules present in solution interacting with similar oppositely charged groups within molecules adsorbed

on the surface. Multilayer adsorption for sugar beet pectin through a similar mechanism has been reported previously (Siew, et al., 2008). Furthermore, it was recently observed that *Acacia senegal* gum alone self associates in solution through electrostatic interaction between protein and carboxylate moieties within its structure as reported in chapter 2.

The adsorbed layer thickness for *Acacia senegal*-NG<sub>1</sub> gum in the presence of 0.5 M NaNO<sub>3</sub> is shown in Figure 3.8 as a function of the concentration of gum and also after various times. Again we see an increase in the adsorbed layer thickness as the concentration of gum is increased and then it reaches a plateau value. The values obtained on Day 0 are significantly greater than for samples prepared in the absence of salt, for example an adsorbed layer thickness of 44 nm was obtained in the presence of 0.05% gum solutions in 0.5 M NaNO<sub>3</sub> compared to 21 nm in water. This value is significantly greater than the value we obtained for the z-average diameter by dynamic light scattering (29.8 nm), but could be accounted for if preferential adsorption of higher molar mass molecules occurred. It is shown in Figure 3.4 above that the radius of gyration,  $R_g$ , for the high molar mass species is typically 20 - 30 nm which is consistent with the dimensions of the AGP reported by Renard et al., 2006, for cylinder-like molecules with a diameter of 59 nm and thickness 5 nm. This indicates that the molecules adsorb end-on at the surface. Snowden et al., (1987),

previously reported that the adsorption capacity for *Acacia senegal* gum adsorbed onto polystyrene latex particles was  $\sim 1 \text{ mg m}^{-2}$  in water and that this increased to  $\sim 5 \text{ mg m}^{-2}$  for adsorption from electrolyte. The increase in the amount adsorbed is explained by the fact that the electrolyte screens lateral electrostatic interactions between the gum molecules adsorbed at the surface allowing more to be accommodated. This behaviour is typical for polyelectrolytes generally. The fact that the adsorbed layer thickness does not increase over time as is the case in water alone can be explained by the fact that the electrolyte would also inhibit protein-carboxylate electrostatic interactions and prevent multilayer adsorption from occurring. This also agrees with the fact that self association of the *Acacia senegal* gum molecules alone in solution was found to be inhibited in the presence of electrolyte (Chapter 2).

Figures 3.9 and 3.10 shows the adsorbed layer thickness for samples prepared with *Acacia seyal* in water and in the presence of 0.5 M  $\text{NaNO}_3$ . The adsorbed layer thickness is of the order of  $\sim 2 - 3 \text{ nm}$  in water and  $\sim 0.5 \text{ nm}$  in 0.5 M  $\text{NaNO}_3$  which is much less than the average hydrodynamic diameter of the gum molecules of 34.2 nm obtained from dynamic light scattering measurements. If we assume that the gum has a similar disk or cylinder-like molecular characteristics to *Acacia senegal* gum this suggests that the gum molecules lie flat on the surface of the particles rather

than end-on. *Acacia seyal* has a lower protein content than *Acacia senegal* and the protein is less accessible as evidenced by the fact that it is not completely hydrolysed by proteolytic enzyme hence the molecules will have a lower affinity for the hydrophobic surface (Flindt et al., 2005). The previous chapter (Chapter 2) has also shown that *Acacia seyal* does not self associate in solution as is the case for *Acacia senegal* and this is attributed to the fact that it has less protein present and also that it is less accessible.

#### **3.4.4. Emulsification properties**

The droplet size distributions of the emulsions prepared with *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gums are presented in Figures 3.11 and 3.12 respectively. It is noted that the droplet size for the freshly prepared emulsions is smaller for *Acacia senegal*-NG<sub>1</sub> than for *Acacia seyal*. In addition, while the droplet size for the emulsion prepared using *Acacia senegal*-NG<sub>1</sub> remains constant on storing for 1 week, the droplet size for the emulsion prepared using *Acacia seyal* increases. This is as expected since it is well known that *Acacia senegal* has superior emulsification properties compared to *Acacia seyal* (Flindt et al., 2005). The fact that *Acacia seyal* is able to form emulsions at all indicates that it must adsorb at the oil-water interface to some degree.

### 3.4.5. Amount of gum Adsorbed

The GPC RI elution profiles for the gum samples and for the supernatants recovered after emulsification are presented in Figures 3.13 and 3.14. The amount of gum adsorbed onto the emulsion oil droplets was calculated from the difference in the areas of the GPC RI elution profiles and was found to be 10.4 mg and 6.9 mg for *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* respectively which corresponds to surface coverages of 3.0 and 2.4 mg m<sup>-2</sup>. These values are consistent with values previously reported for the adsorption of *Acacia senegal* gum onto limonene droplets (Padala, Williams and Phillips, 2009). These Figures also show that for *Acacia senegal*-NG<sub>1</sub> gum there is preferential adsorption of high molecular mass gum eluting at 7 - 10 mL and also lower molecular mass material eluting at 13 - 15 mL. These correspond to the AGP and GP respectively, in Figures 3.2 and 3.3. For *Acacia seyal*, there was no apparent preferential adsorption of high molecular mass species but rather adsorption occurred across the whole molecular mass range.

### 3.4.6. Transmission electron micrographs

Electron micrographs of the polystyrene latex particles in the presence and absence of the *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gums are presented in Figures 3.15 - 3.17. From Figure 3.15, it can be observed that the latex particles in the absence of either of

the *Acacia* gum exudates seems to have been stained by the uranyl acetate, which is probably due to the fact that the stain is cationic in nature. It is also apparent that the latex particles are polydisperse and display a variety of sizes. Figure 3.16 confirms that the *Acacia senegal*-NG<sub>1</sub> gum molecules have formed a distinct, thick adsorbed layer surrounding the particles while, Figure 3.17 demonstrates the presence of a thinner, more diffuse layer around the particles. These observations support the findings reported above for the amount of gum adsorbed.

The thickness of the adsorbed layer of *A. senegal*-NG<sub>1</sub> gum (Figure 3.16) is approximately 15.5 nm which is in reasonable agreement with the value of 21 nm recorded on day 1 in a 0.05% (w/w) solution in water from the dynamic light scattering measurements. The slight difference is most likely due to the fact that the adsorbed layer observed by negative staining has been air-dried prior to imaging and any residual water will be removed once subjected to the high vacuum present within the entry chamber of the transmission electron microscope. As such, the observed thickness of the adsorbed layer is in close agreement with the thickness recorded by dynamic light scattering.

In direct contrast, the adsorbed layer on the latex particles in the presence of *A. seyal* gum (Figure 3.17) is much less distinct and is thinner, which is also consistent with the fact that the adsorbed

layer thickness values obtained by dynamic light scattering was  $\sim 2$  - 3 nm.

### 3.5.0 Summary

In this chapter, study of the emulsification properties of *Acacia* gum of *A. senegal*-NG<sub>1</sub> and *A. seyal* samples was carried out, so as to obtain a clearer understanding of the mechanisms underlying the differences in the emulsification properties demonstrated by gum exudates produced by the species of *A. senegal* and *A. seyal* plants. The result revealed that *A. seyal* gum was a worse emulsifier than the *A. senegal*-NG<sub>1</sub> gum and was slower to aggregate.

The droplet size of gum Arabic oil-in-water emulsions prepared from the samples was determined and the result demonstrated that the Nigerian *Acacia senegal*-NG<sub>1</sub> gum sample droplet size, was smaller than that obtained for the *Acacia seyal* sample. Furthermore, while the droplet size for the emulsion prepared using the *Acacia senegal*-NG<sub>1</sub> sample remained constant upon storage (for 1 week), the droplet size for the emulsion prepared using *Acacia seyal* increased.

The hydrodynamic size of the molecules present in both the *A. senegal*-NG<sub>1</sub> and *A. seyal* gums in an aqueous state was also monitored as a function of time, and it was found that molecular



association/aggregation occurred and was inhibited in the presence of an electrolyte (0.5 M NaNO<sub>3</sub>).

The adsorbed layer thickness of the gum Arabic adsorbed onto polystyrene latex particles was then determined, the thickness of the adsorbed layer was shown to increase as the amount of gum present increased. The adsorbed layer thickness also increased significantly with time. As for samples prepared with *Acacia seyal* in water and in the presence of 0.5 M NaNO<sub>3</sub>, the adsorbed layer thickness is much less than that of the *Acacia senegal*-NG<sub>1</sub> gum.

## **4.0. CHAPTER FOUR**

### **4.1.0. Electron microscopy analysis of *Acacia* gum exudate from *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal***

#### **4.1.1. Introduction**

*Acacia* gum exudates are highly heterogeneous substances which contain both a variation in monomer composition and/or in the linking and branching of the monomer units. They also vary in the molecular mass distribution of the component macromolecules, both in terms of polysaccharides and glycoproteins/proteoglycans. The consequence of this heterogeneity is reflected in the variety of molecular species collected after fractionation of the gum, which changes according to both the mode of separation and the method of detection used.

As described in earlier chapters, three main fractions/components can be isolated from the gum using one of the most widely used fractionation methods i.e. hydrophobic interaction chromatography/gel permeation chromatography (Randall, Phillips, and Williams, 1989). Analysis of these three fractions demonstrated that each contained similar proportions of a variety of monosaccharides, although they differed in their molecular masses, protein content and amino acid composition. The proteinaceous component of the first two fractions had similar amino acid distributions, with hydroxyproline and serine being the most abundant. Whereas, the amino acid composition of the third

(GP) fraction was significantly different, with aspartic acid being the most abundant (Randall, Phillips and Williams, 1989; Renard et al., 2006).

The bulk of the gum (~88% of the total weight) was shown to be comprised of an arabinogalactan peptide (AG). The second major fraction (~10% of the total weight) was identified as an arabinogalactan-protein (AGP) complex. The third minor fraction (~1%) consists of a glycoprotein (GP) complex.

With regards previous analyses of the molecular structure of molecules present in each of the fractions, Sanchez and his colleagues showed by transmission electron microscopy and atomic force microscopy that the AG fraction consisted of oblate ellipsoids with a ~20 nm diameter and ~1.5 nm thickness with an inner, interspersed chain network (Sanchez et al., 2008).

In terms of the AGPs, these molecules were first described in terms of a “wattle-blossom” macromolecular assembly by virtue of a few (~5) discrete polysaccharide domains of  $M_w \sim 2 \times 10^5$  kDa held together by a short core protein (Fincher, Stone, and Clarke, 1983).

An alternative model to describe the structure of molecules present in the AGP fraction was proposed by Qi, Fong and Lamport, (1991), who isolated the AGP fraction by preparative GPC and subjected it to deglycosylation using hydrofluoric acid. They found that the resultant core protein consisted of ~400 amino acids, containing

~120 hydroxyproline residues with a 10 - 12 residue repetitive motif. Observation of the polypeptide using transmission electron microscopy indicated that it was ~150 nm long, 5 nm diameter and that it was rod-like in shape. The authors concluded that the molecules observed resembled a 'twisted hairy rope' with an axial ratio of ~30:1 (Qi, Fong and Lamport, 1991). This model would be consistent with the fact that large macromolecules, like AGPs, could migrate through a primary cell wall with 4 to 5 nm porosity, by reptation (Carpita, 1982).

However, a rod-like configuration for the gum Arabic AGP is inconsistent with the light scattering and viscometric data, which indicate a more compact conformation (Idris, Williams and Phillips, 1998; Siddiq, et al., 2005). The linear molecules observed may also adopt a different conformation in solution and TEM analysis of purified AGPs from other species have indicated a compact, "wattle-blossom"- like shape to these molecules (Baldwin, McCann and Roberts, 1993; Cheung, Wang and Wu, 1995).

Subsequent to which, Mahendran, et al., (2008) identified smaller carbohydrate blocks of  $\sim 4.5 \times 10^4$  Da linked by O-serine and O-hydroxyproline residues to a polypeptide chain of approximately 250 amino acids in length (~30 kDa core protein), present in the AGP fraction. On the basis of which, the authors proposed a structural model where the shape of the macromolecule would be a kind of spheroidal random coil, consistent with the "wattle-

blossom" model proposed by Fincher (Fincher, Stone and Clarke, 1983). This spheroidal, random coil would be composed of a folded polypeptide chain bearing large sugar blocks with a possible thin, oblate, ellipsoid morphology (Mahendran et al., 2008).

More recently, Renard et al., (2012) reported that the AGP present in the gum when in solution had two types of conformation, with different molecular weights. The low molecular weight population, with long-chain branching had a compact structure, while the high molecular weight population with short chain branching had a more elongated conformation (aggregates of the smaller molecules). The thicknesses of these two populations were below  $\sim 5$  nm and approximately 85% of the particles observed displayed an apparent diameter 20 - 80 nm. Single molecules with a spheroidal shape and aggregated molecules with an elongated shape were both reported to possess an outer structure combined with an inner porous network of interspersed chains or interacting structural blocks.

With regards studies of the morphology of the molecular components of the glycoprotein fraction, Renard et al., (2014) indicated that when in solution this fraction also consisted of a mixture of single and aggregated molecules. These molecules displayed a high propensity to self-associate in to either linear or circular ring structures. There was no outer structure associated to the inner porous network of interspersed chains observed in the

spheroidal single molecule. The structure of the single molecule was that a thick shell wrapped a central hole and gave rise to a ring-like morphology with  $\sim 8 - 11$  nm diameters.

#### **4.1.2. Self-aggregation of gum Arabic molecules**

The macromolecules present in gum Arabic have a tendency to self-aggregate as has been noted in the literature (Renard et al., 2006; Sanchez et al., 2008; Renard et al., 2012; Renard et al., 2014; Gashua et al., 2015). Renard et al., (2006) considered that these molecular associations were through hydrogen or disulphide bonds. This phenomenon was described by Li et al., (2009) using rheological and dynamic light scattering. These authors revealed that this aggregation had an influence on the rheological behaviour of the gum in solution.

Molecular association is known to affect the performance of gum Arabic in solution due to the influence of molecular weight, shape and size. (Al-Assaf, et al., 2009; Renard et al., 2014). Furthermore, Al-Assaf, et al., (2009) studied the role played by the proteinaceous components in *Acacia* gums to promote associations when the gum is subjected various processing treatments such as maturation, spray drying and irradiation. Their results revealed that the ability of the proteinaceous components to promote hydrophobic association is directly influenced by the size and proportion of the AGP(s) present. The molecular association of the

gum in aqueous solution has also been observed using a variety of analytical techniques which include small angle X-ray scattering (SAXS), cryotransmission electron microscopy, light scattering and rheological methods (Dror, Cohen and Yerushalmi-Rozen, 2006; Wang et al., 2008; Li et al., 2009).

As previously described in chapter two, gum Arabic harvested from *A. senegal*-NG<sub>1</sub> is thought to associate in aqueous solution through electrostatic interaction between glucuronic acid and protein residues present on different molecules, thus forming electrostatic complexes, which is also the case for the *Acacia seyal* gum exudate. The observed increase in hydrodynamic size is directly proportional to the increase in protein content, whilst the hydrodynamic size was not observed to change in the presence of 0.1 M NaNO<sub>3</sub>. A similar observation was made by Williams et al (2012), which supports the hypothesis that the presence of an electrolyte would screen the electrostatic interactions between the gum component macromolecules.

This phenomenon negatively affects its emulsification properties as well its rheological behaviour which is of direct relevance to its multitude of applications in the food industry.

#### **4.1.3. Aims and objectives**

The main objective of the work presented in this chapter, was to obtain a preliminary understanding of the molecular structure/composition of two selected *Acacia* gum exudates harvested from *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* using transmission electron microscopy (TEM). The second objective was to investigate aggregation of these molecular components over time, using TEM and scanning transmission electron microscopy (STEM).



## **4.2.0. Materials and methods**

### **4.2.1. Materials**

#### **4.2.1.1. Gum samples**

The two samples of gum Arabic used for the various microscopic analyses described in this chapter, were the Nigerian *Acacia senegal* gum sample (NG<sub>1</sub>) and the Sudanese *Acacia seyal* gum sample described previously in chapter two, both of which were analysed in their 'raw' untreated form.

#### **4.2.1.2. Antibodies**

An anti-cell wall/plasma membrane rat monoclonal antibody (JIM8) was employed for the immunogold labelling experiments. The JIM8 binds to a terminal sugar epitope present on arabinogalactan-proteins and arabinogalactans (Pennell et al, 1991; Huang et al., 2013). This antibody was kindly provided by Professor Paul Knox (University of Leeds).

## **4.2.2. Methods**

### **4.2.2.1. Transmission Electron Microscopy**

#### **4.2.2.2. Negative staining**

Solutions of 1% (w/w) *A. senegal*-NG<sub>1</sub> and *A. seyal* gum samples were prepared in sterile deionised water.

Formvar/carbon-coated nickel (TEM) grids (200 mesh) were incubated on the surface of 30 µl drops of the sample solutions for 90 seconds. Excess liquid was carefully removed by touching the edge of the grids on to filter paper (wick).

The grids were then negatively stained by placement on a 30 µl drop of 2% (w/v) Uranyl acetate for 90 seconds. Excess Uranyl acetate was removed from the grids as described above. The negatively stained grids were then air dried, prior to being observed on a JEOL JEM-1200 EX Transmission Electron Microscope, at an accelerating voltage of 80 kV. The images were photographed using a GATAN retractable multi scan camera.

#### **4.2.2.3. Immunogold negative staining**

For the immunogold negative staining experiments, 1% (w/w) solutions of each sample were prepared in sterile deionised water as previously described. Formvar/carbon-coated nickel TEM grids (200 mesh) were first washed by floatation on 30 µl drops of TBS for 5 minutes. The grids were then dried by wicking excess liquid on to a piece of filter paper. The grids were incubated on 30 µl

drops of the gum samples (which serve as the antigen) for 5 minutes. The grids were then wicked dry, followed by incubation on 30  $\mu$ l droplets of ammonium acetate for 1 minute. Before being transferred onto the blocking agent (10% w/v Fetal Calf Serum dissolved in TBS) for 30 minutes. The grids were dried and incubated on 30  $\mu$ l of undiluted primary antibody (JIM8) for 30 minutes (negative controls were not incubated with the primary antibody). The grids were dried and washed 6 times, 5 minutes per wash on successive drops of sterile distilled water, during which process the grids were wicked dried between each wash. Grids were then incubated on 30  $\mu$ l of a 1:30 dilution of a gold conjugated secondary antibody (12 nm Colloidal Gold-Affinipure Goat Anti-Rat IgG) for 30 minutes. After which the grids were wicked dried and washed for 5 minutes per wash on 6 droplets of sterile distilled water. They were then negative stained by placement on 30  $\mu$ l droplets of 2 % (w/v) Uranyl acetate for 90 seconds then air dried prior to observation on a JEOL JEM-1200 EX Transmission Electron Microscope, at an accelerating voltage of 80 kV. The images were photographed using a GATAN retractable multi scan camera.

#### **4.2.2.4. Aggregation experiments**

Solutions of 1% (w/w) samples were prepared with sterile distilled water for the aggregation experiments. The experiment was performed to study change in molecular size and shape of the gum Arabic molecules in aqueous solution over time. The sample solutions were incubated at room temperature in a stationary position, for a period of 5 days. Observations were recorded on day 0, day 1 and day 5.

For the day 0 observation, 30  $\mu$ l of each sample solution was applied to a formvar/carbon-coated TEM nickel grid (200 mesh) for 90 seconds immediately after preparation of the solutions then air dried. The grids were negatively stained for 90 seconds with 2% (w/v) uranyl acetate as described previously. They were then air dried prior to observation. The same procedure was followed after 1 day and 5 days incubation.

#### **4.2.2.5. Scanning transmission electron microscopy (STEM)**

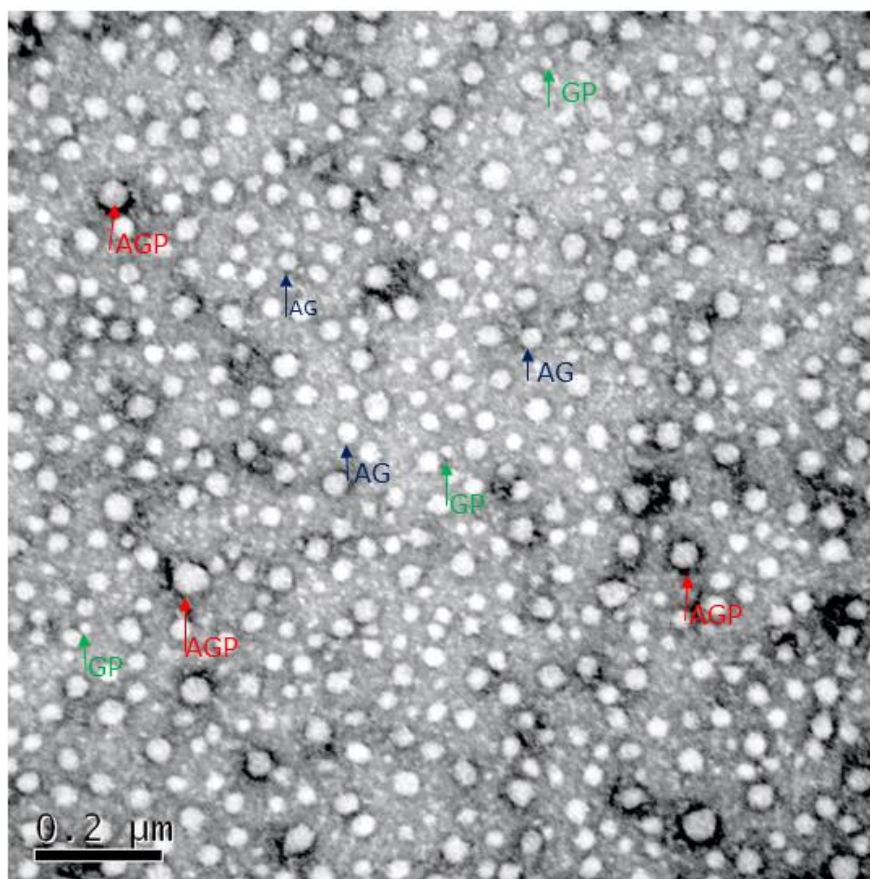
Scanning transmission electron microscopy (STEM) imaging was performed in a JEOL 7000F SEM using the transmitted electron detector. The work was performed at 20 kV.

Samples were prepared as described previously on formvar/carbon-coated Nickel 200 mesh grids.

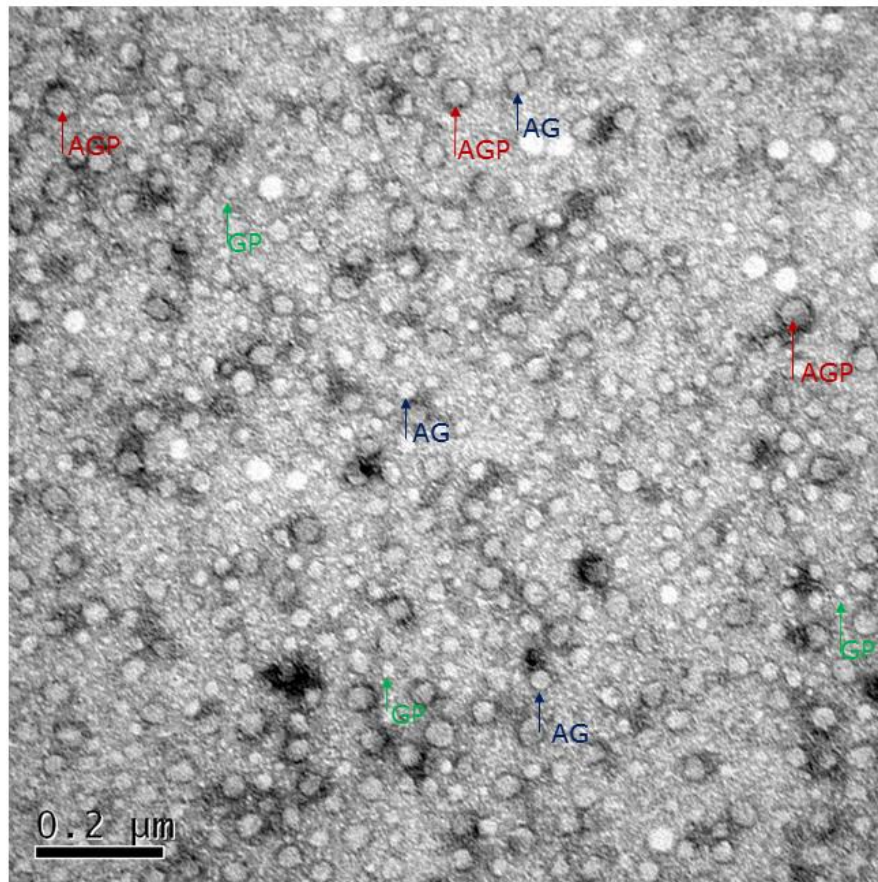
### 4.3.0. Results

#### 4.3.1. Negative staining

The results of the negative staining experiments using the two gum samples (*Acacia senegal*-NG<sub>1</sub> and *Acacia seyal*) are presented in figures 4.1. and 4.2. below.



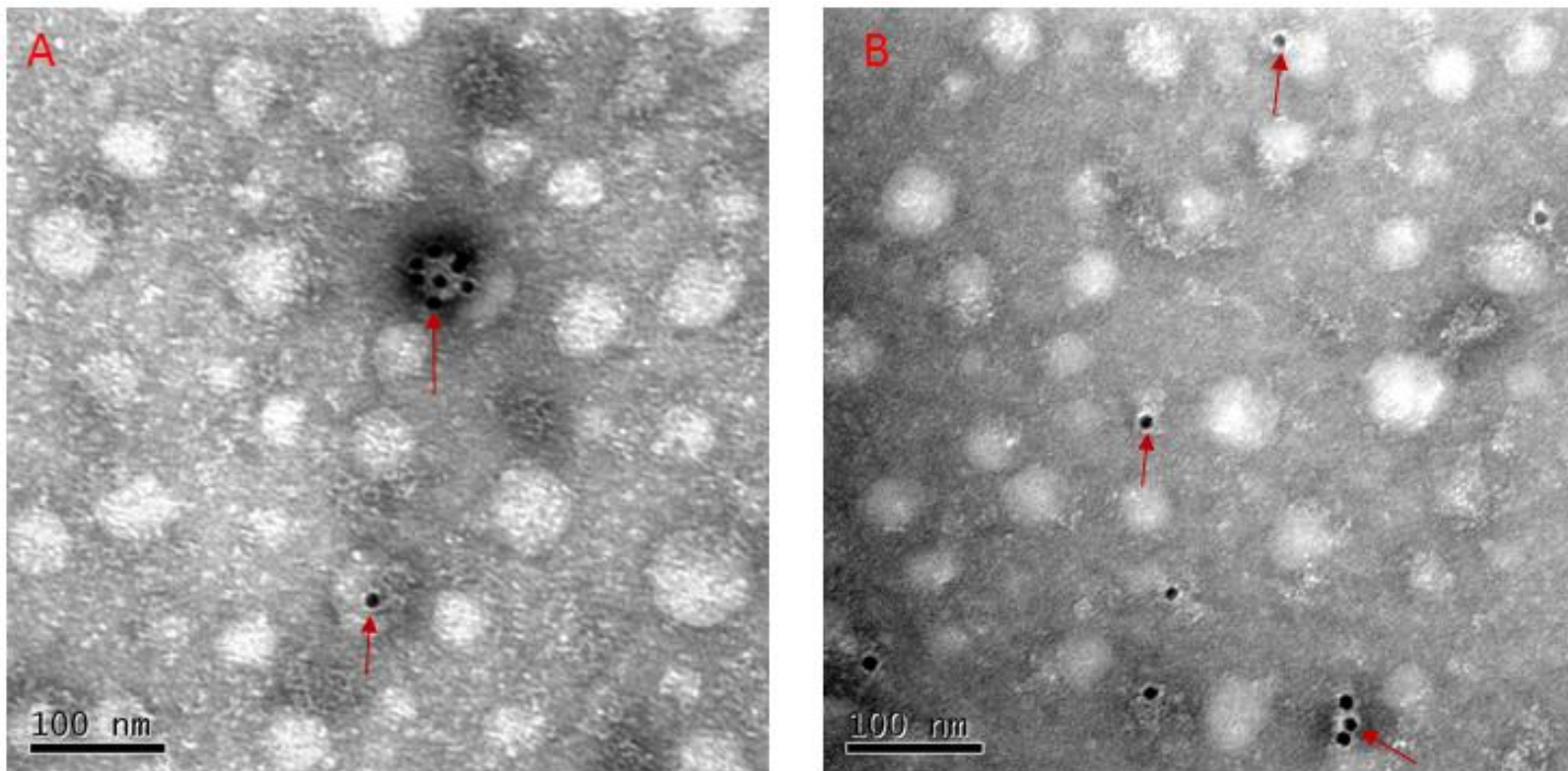
**Figure 4.1.** Transmission electron micrograph of *Acacia senegal*-NG<sub>1</sub> gum (1% w/w) negatively stained, showing the distribution of molecules present in the sample. The red arrows indicate molecules of ~60 nm, which are putative AGP molecules, and the blue arrows indicate molecules of ~20 nm (putative AG) while the much smaller molecules of ~12 nm (putative GP) are indicated by the green arrows.



**Figure 4.2.** Transmission electron micrograph of *Acacia seyal* gum (1% w/w) negatively stained, showing the distribution of molecules present in the sample. The red arrows indicate molecules of  $\sim 60$  nm, which are putative AGP molecules, and the blue arrows indicate molecules of  $\sim 20$  nm (putative AG) while the much smaller molecules of  $\sim 12$  nm (putative GP) are indicated by the green arrows.

#### **4.3.2. Immunogold negative staining**

Immunogold negative staining was used with both gum samples in order to identify plant cell wall-associated epitopes present in the samples. An antibody, JIM8 was selected for this work. The result is presented in Figure 4.3.

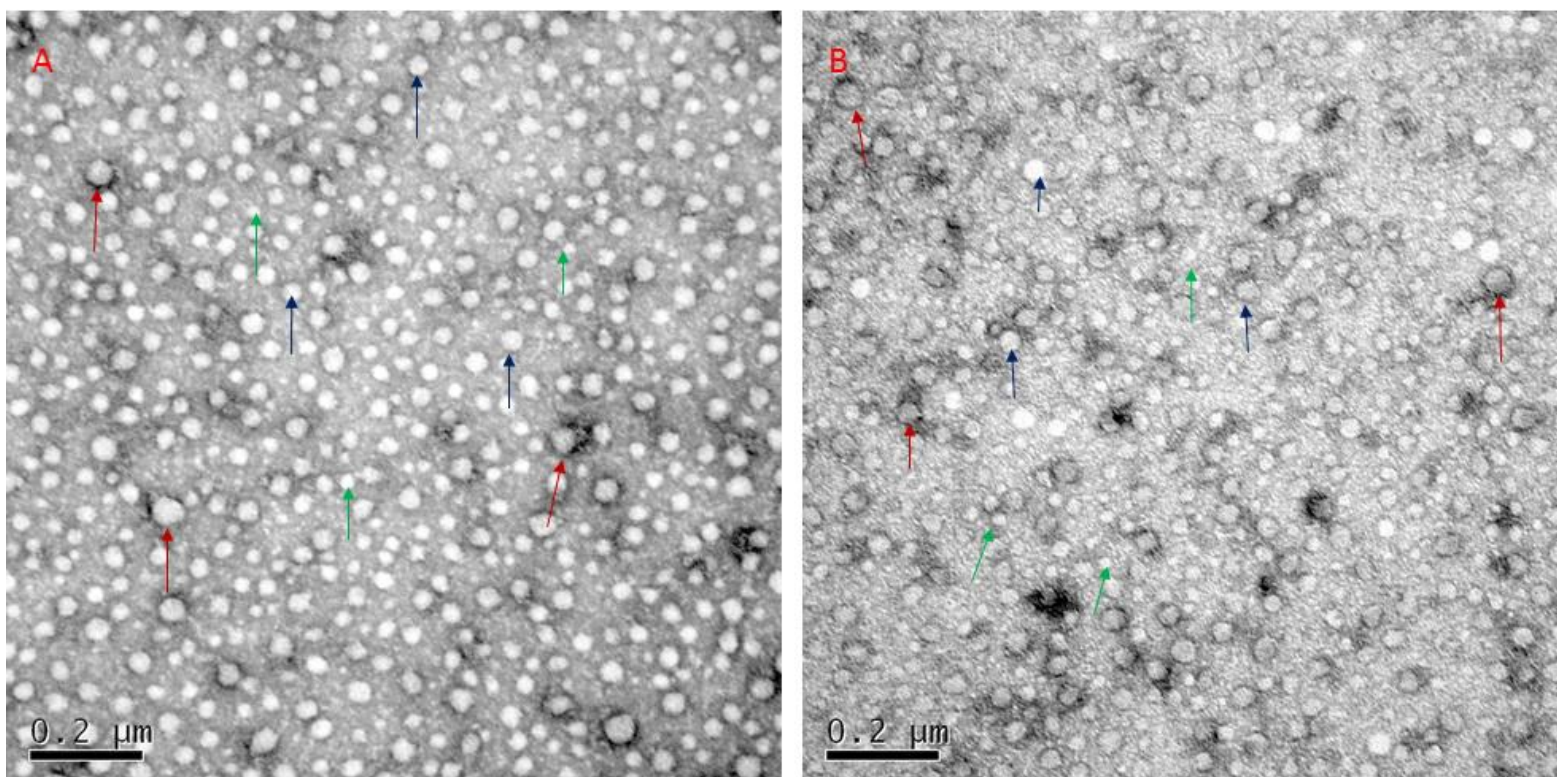


**Figure 4.3.** Transmission electron micrographs of *Acacia senegal*-NG<sub>1</sub> (A) and *Acacia seyal* (B) gum samples labelled with JIM8 monoclonal antibody. The red arrows in A and B indicated the gold particles which appear as black dots on the labelled molecules, indicating that they are labelled with the antibody. Therefore JIM8 clearly labels molecules present in the *gum* samples. Scale bars = 100 nm.

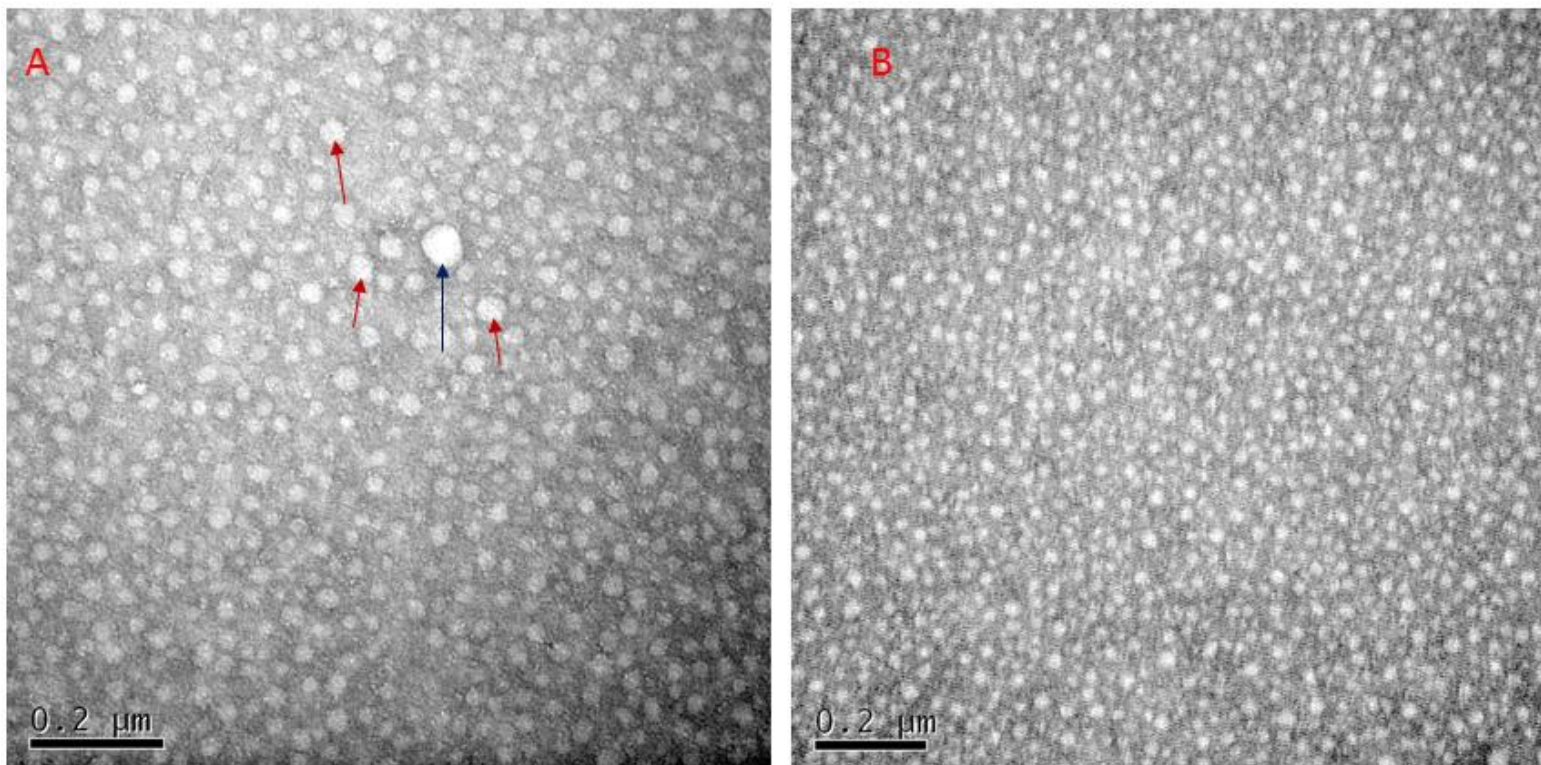


### **4.3.3. Molecular aggregation**

The degree of aggregation of molecules in aqueous solutions of both *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gum samples were monitored over a period of five days so as to assess their level of aggregation over time using TEM and STEM. The results for the transmission electron microscopy are presented in Figures 4.4 - 4.6 for day 0, day 1 and day 5, while the scanning transmission electron microscopy result for day 5 is presented in Figure 4.7.

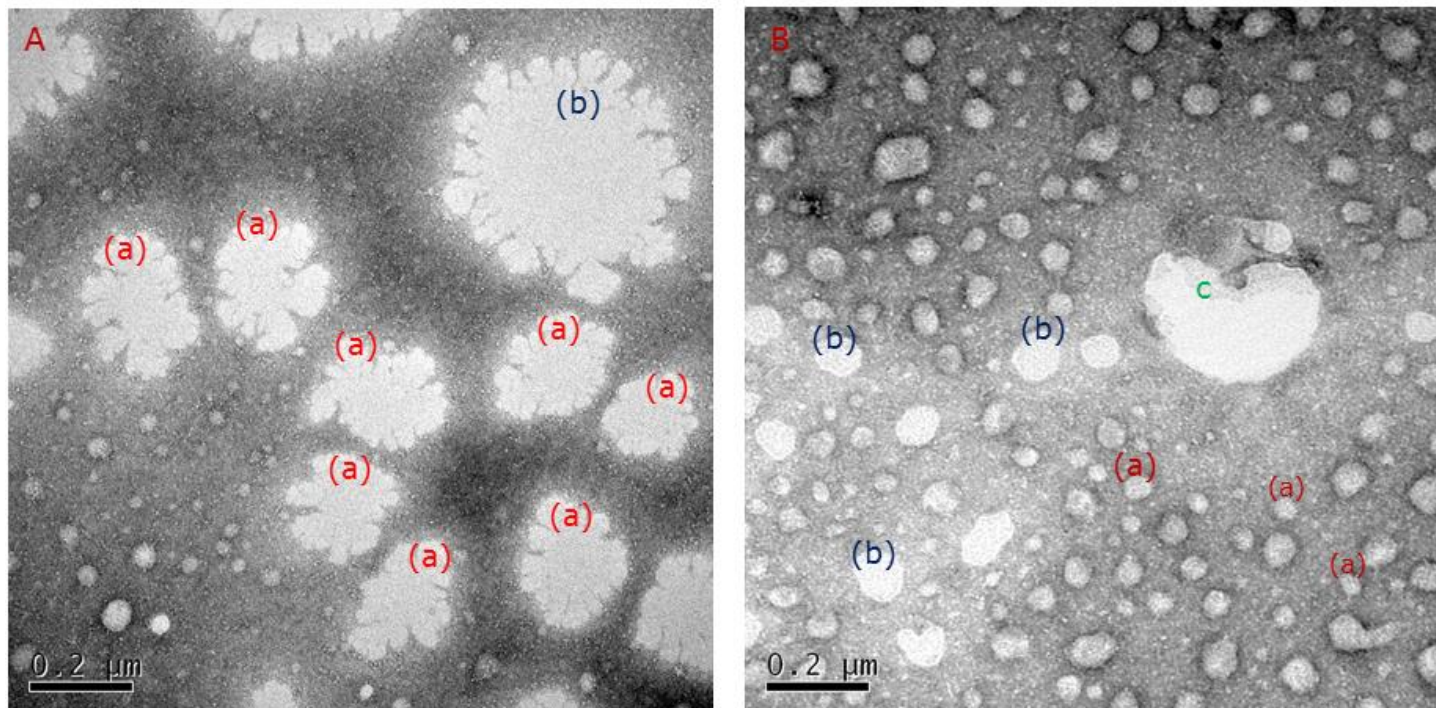


**Figure 4.4.** Transmission electron micrographs of *Acacia senegal*-NG<sub>1</sub> (A) and *Acacia seyal* (B) gums, negatively stained at day 0. Single molecules of varied sizes are observed in both gum samples as indicated by the coloured arrows. The red arrows indicates molecules of ~60 nm (putative AGP), and the blue arrows indicates molecules of ~20 nm (putative AG), while the much smaller molecules, are indicated by the green arrows which represents the molecules of ~20 nm (putative GP). (Scale bar = 0.2 μm).



**Figure 4.5.** Transmission electron micrographs of *A. senegal*-NG<sub>1</sub> (A) and *Acacia seyal* (B) gums negatively stained and observed after 1 day. Aggregation is observed to have started to occur in the *A. senegal*-NG<sub>1</sub> sample (A) with a number of ~40 nm aggregates observed, (as indicated with the red arrows) and a larger aggregate of ~66 nm (indicated with the blue arrow). However, there is no apparent aggregation present in the *A. seyal* gum sample at this stage. The scale bar = 0.2 μm in both A and B.

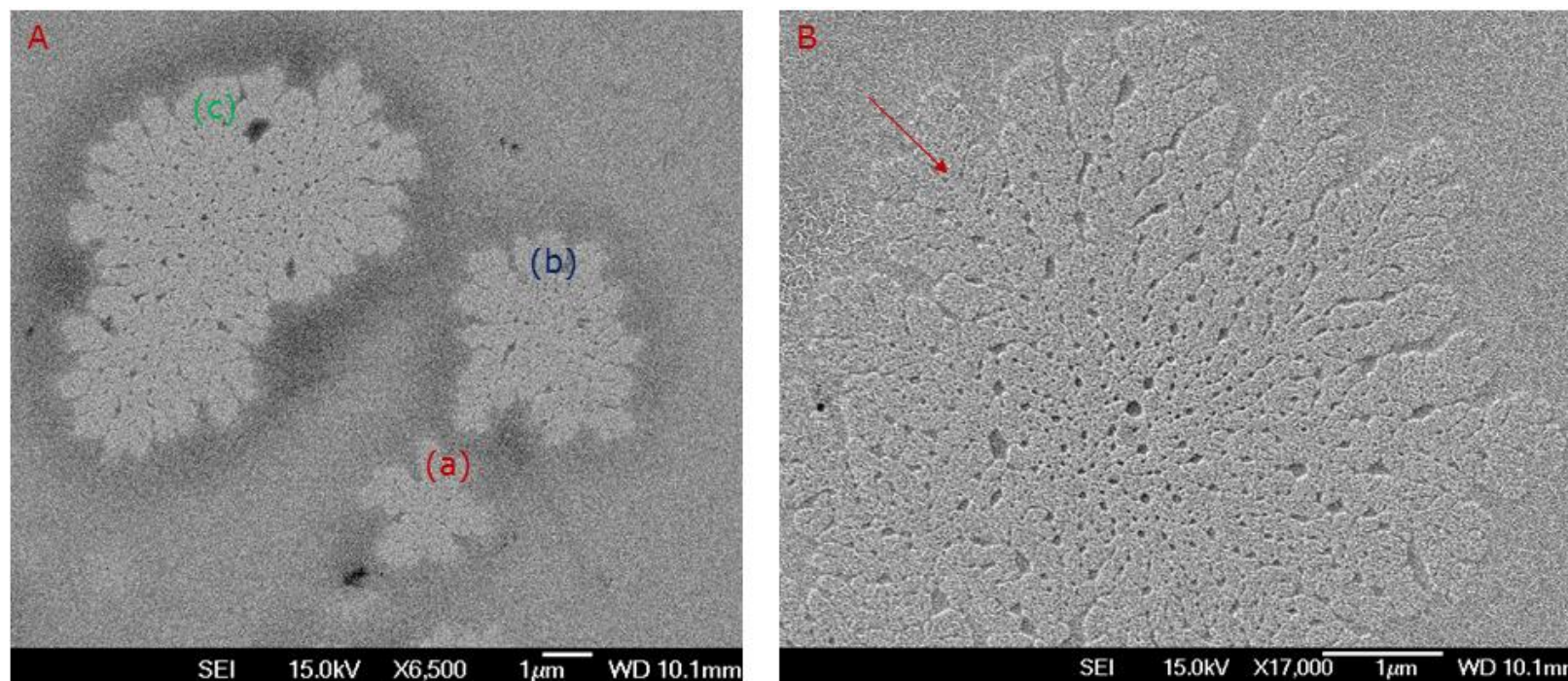




**Figure 4.6.** Transmission electron micrographs of *Acacia senegal*-NG<sub>1</sub> (A) and *Acacia seyal* (B) gums negatively stained and observed after 5 days. Distinct snowflake-like aggregates were observed in the *Acacia senegal*-NG<sub>1</sub> gum in varied sizes with an average diameter of ~184 nm (a), with another larger aggregate of ~420 nm (b). The *Acacia seyal* gum also contains aggregates, with average diameter range of ~37 nm (a) and ~80 nm (b), with a larger aggregate of ~320 nm (c), but the aggregated are not snowflake-like manner, they seem to be more ellipsoidal in shape. The scale bar = 0.2 μm in both A and B.

#### **4.3.4. Scanning Transmission Electron Micrographs**

The images of *Acacia senegal*-NG<sub>1</sub> obtained using the scanning electron transmission microscope (STEM) are presented in Figure 4.7 below. The Images are taken at two different magnifications as shown in the figure, with A= X6, 500 and B= X17, 000. Larger aggregated molecules were observed. This is to further elucidate the structure of the molecules. The structure of aggregated molecules is clearly visible using this technique which confirms the structure observed using the negative staining of TEM.



**Figure 4.7.** Scanning transmission electron micrographs of *Acacia senegal*-NG<sub>1</sub> sample after incubation for 5 days at room temperature. Images taken at two magnifications are shown A, X6, 500 and B, X17, 000. Aggregates of  $\sim 2\ \mu\text{m}$  (a) and  $\sim 3\ \mu\text{m}$  (b) and larger aggregated molecules of  $\sim 6\ \mu\text{m}$  were observed in A. The Image B is an enlargement of (b). This is to further elucidate the structure of the molecules. The snowflake-like structure of aggregated molecules is clearly visible using this technique which confirms the structure observed using the negative staining of TEM.

#### **4.4.0 Discussion**

##### **4.4.1. Negative staining**

As described in the introduction to this chapter, Sanchez et al., (2008) revealed that the AG peptide fraction of gum Arabic appeared to contain a dispersion of two-dimensional structures with a  $\sim 6.5$  nm gyration radius and an inner dense branched structure using small angle neutron scattering. Based upon transmission electron and atomic force microscopy, a disk-like model with  $\sim 20$  nm diameter and  $\sim 2$  nm thickness for the AG molecules was proposed.

From the current study, it can be observed that both gum samples contain molecules of  $\sim 20$  nm diameter (see Figures 4.1 and 4.2) which may therefore be AG molecules. This observation is also likely to be correct given that AG constitutes  $\sim 90$  % of the gum by weight (Randell, Phillips and Williams, 1989) and in micrographs resulting from our study, molecules of  $\sim 20$  nm are the most abundant, in both samples. Therefore, both the size and abundance of these molecules indicate that they are arabinogalactans/arabinogalactan peptides.

The molecular structure of arabinogalactan protein (AGP) fraction was studied by Renard et al., (2012). The study revealed the existence of two types of AGP molecules/complexes present in the gum: one with a low molar mass, with long-chain branching and a compact conformation and the other with a high molar mass,

short-chain branch and an elongated conformation. About 85% of molecules in the AGP fraction were observed to have a diameter between  $\sim 20$  nm and  $\sim 80$  nm. The remainder had a diameter ranging from  $\sim 80$  nm to  $\sim 120$  nm which most likely corresponded to elongated aggregates of the smaller particles.

Both of the samples used in this study were shown to contain a high quantity of molecules of  $\sim 60$  nm diameter (Figures 4.1 and 4.2). These have a compact, globular shape. Therefore, from their diameter, it is most likely that these are AGP molecules.

As to the GP fraction, Renard et al., (2014) studied the molecular composition of this fraction using high-performance size exclusion chromatography-multi angle laser light scattering (HPSEC-MALLS), small angle X-ray scattering, synchrotron radiation circular dichroism and transmission electron microscopy. Renard and his colleagues reported that the glycoprotein molecules they observed appeared to form a ring of  $\sim 9$  nm diameter and often self-assembled to form linear or circular "string-of-rings" (aggregates).

The major difference between the GP and the other two fractions was that there was no outer structure combined to an inner porous network of interspersed chains observed in the GP molecules.

In this study, molecules with  $\sim 12$  nm diameter (Figure 4.1) and  $\sim 8$  nm diameter (Figure 4.2) were observed in the midst of the more frequently observed  $\sim 20$  nm (AG) and  $\sim 60$  nm (AGP) particles



these values are similar to those reported by Renard for the glycoprotein component(s) of the gum.

#### **4.4.2. Immunogold negative staining**

Upon completion of the negative staining experiments, immunogold negative staining was performed using the anti-cell wall/plasma membrane antibody (JIM8) in order to attempt to identify cell wall-associated epitopes present in the two gum samples.

JIM8 binds to a terminal sugar epitope present on arabinogalactan-proteins and arabinogalactans (Pennell et al, 1991; Huang et al., 2013).

From the results, the two samples were labelled with the JIM8 primary antibody (Figures 4.3 A and B). Several molecules are clearly labelled in the grids. Moreover, the molecules labelled by JIM8 are those with a diameter of ~60 nm which were identified in the negative staining experiments as putative AGPs. Therefore, the negative staining results in combination with the immunogold labelling (using the JIM8 antibody) strongly suggest that the molecules of ~60 nm present in the two samples are indeed AGPs. These data represent the first direct immunological evidence to support the previous work by Renard and his colleagues (Renard et al., 2012).

#### 4.4.3. Molecular self-aggregation

Gum Arabic in solution is known to form aggregates (Evans, Ratcliffe and Williams, 2013). Many food-grade proteins and polysaccharides aggregate when heated, including gum Arabic aggregate (Aoki et al., 2007; Al-Assaf et al., 2009). In the current study, gum Arabic solutions were incubated at room temperature (25 °C) for 0 day, 1 day and 5 days in order to observe the aggregation process in the two gum samples. From the results (Figures 4.4, 4.5 and 4.6), it can be seen that at 0 day (Figure 4.4), the molecules present in both the *Acacia senegal*-NG<sub>1</sub> and *Acacia seyal* gum samples are not aggregated and single molecules can be clearly observed. However, by day 1, some small aggregates have formed in the *A. senegal*-NG<sub>1</sub> sample (see Figure 4.5). By 5 day, from (Figure 4.6), large aggregates with ~184 nm average diameter were observed in the *A. senegal*-NG<sub>1</sub> sample together with a larger aggregate of ~420 nm (A). In the *A. seyal*, similar trend was observed, but with the molecules smaller than in *A. senegal* sample. An average of ~37 nm and ~80 nm were observed together with a larger ~320 nm aggregate (B). This could be attributed to the nature of interaction of the molecules in the different gums due to the amount and accessibility of the protein components of the different gums as discussed in chapter three. Similar observation was made by Williams, (2012), in which multilayers were reported to be formed as a result of aggregation

of molecules due to interaction in solution. In the case of *Acacia seyal* gums, it shows less aggregation in solution. This is because, its protein is less accessible and therefore have a lower affinity for hydrophobic surfaces as reported previously by Flindt et al., (2005).

#### **4.4.4 Scanning transmission electron microscopy**

The STEM micrographs of the 5 day old *Acacia senegal*-NG<sub>1</sub> gum aggregates are presented in Figure 4.7. The snowflake-like structure of the aggregated gum molecules using this technique confirm the structures observed in the negatively stained TEM micrographs of aggregated molecules after 5 days of incubation at room temperature (Figure 4.6).

#### **4.5.0. Summary**

To summarise, the data presented in this chapter represent a preliminary microscopic analysis of the molecular structure/composition of the two selected gum samples (*A. senegal*-NG<sub>1</sub> and *A. seyal*) and the aggregation of these molecules over time. Further immunogold and STEM experiments will be required to follow up on the very interesting data presented, and to broaden the scope of the study.

## **5.0. Chapter Five**

### **5.1.0. General Discussion**

#### **5.1.1 Introduction**

Gum Arabic harvested from *Acacia senegal* and *Acacia seyal* is an approved food additive (E414), where it is mainly used to inhibit sugar crystallization and is also widely used as an emulsifier in the industry (Osman et al., 1993; Rodger, et al 2012). Furthermore, it has a wide variety of other industrial applications which include, as an additive encapsulator in the textile, lithography, cosmetic and pharmaceutical industries (Adeleye, Odeniyi and Jaiyeoba, 2011; Ikoni and Ignatius, 2011).

As stated in the introduction to this work (Chapter one), *Acacia senegal* is an important agroforestry cash crop in several sub-Saharan countries including Nigeria (world's second largest producer of gum Arabic). Moreover, it is clear from the literature that very few studies have been carried out on *Acacia* gums known to have been harvested in Nigeria and next to nothing on their characterisation. Furthermore, since it is well known that the chemical composition of *Acacia* gums (which determine their value) varies with geographical location/origin, weather conditions, soil type and the genotype of plants from which the gum is harvested and since Nigeria is the world's second largest supplier of the gum (Idris, Williams and Phillips, 1998; Yebeyen, Lemenih and Faleke., 2009; Hababella, Hamza and El Gaali, 2010; Harmand, 2010); it

was thought timely to investigate and compare the physicochemical characteristics of gum exudate samples harvested from mature trees of *Acacia senegal* at two different specific ecolocations in Nigeria, together with well-known previously characterised gum samples harvested from *Acacia senegal* and *Acacia seyal* originating from Sudan. As such, this investigation was of clear agronomic importance in relation to the use of Nigerian gum Arabic in the food industry, and the expansion of the Nigerian gum industry in the future.

#### **5.1.2. Main Findings**

For the monosaccharide sugar composition analyses of the four gum samples used in this study, a method previously described by Yadav et al., (2007), using high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD), and methanolysis combined with trifluoroacetic acid (TFA) hydrolysis was used.

The results of the study have shown that the *A. seyal* gum had a lower rhamnose and glucuronic acid content than the *A. senegal* samples, but had a higher arabinose content as has been reported previously (Anderson, et al., 1990; Williams and Phillips, 2009). No significant differences were observed between the sugar compositions of the *A. senegal* gums from Sudan and Nigeria.

The protein content of the samples was determined by the Kjeldahl method, as approved by the Association of Analytical Communities, (1999) which involved catalytic digestion of sample with H<sub>2</sub>SO<sub>4</sub>. The amino acid composition was then measured using the hydrolysis method based on modular HPLC components controlled by a NASCOM III system. The result of these analyses showed that the total protein content of the Nigerian gum samples was significantly higher than recorded for the Sudanese samples. This may relate to many factors including the genotype of the trees from which the gums were harvested, the age of the trees, the soil and exposure to biotic and abiotic stressors (Idris, Williams and Phillips, 1998; Karamalla, Siddig and Osman, 1998). The principal amino acids present in all the gum samples were hydroxyproline, serine, aspartame, threonine and proline which is in agreement with literature values (Anderson, et al., 1990).

Gel permeation chromatography of the samples coupled to light scattering, refractive index and UV detectors, showed the presence of arabinogalactan, arabinogalactan-protein and glycoprotein fractions and also shown the presence of an additional small proportion of very low molar mass proteinaceous material in all the samples which has previously been ignored. The plot of radius of gyration,  $R_g$ , as a function of elution volume showed a discontinuity for one of the Nigerian samples and for the *A. seyal* gum sample at elution volumes corresponding to the AGP component suggesting a

different molecular structure. Plots of  $M_w$  versus  $R_g$  confirmed that the molecules had a compact structure.

In recent years, there has been increased interest in the use of gum Arabic as a dietary fibre and its application as an emulsifier for the encapsulation of active compounds, (Vinayahan, Williams and Phillips, 2012; Evans, Ratcliffe and Williams, 2013). Therefore, a better understanding of the degree to which gum Arabic is adsorbed during the formation of stable emulsions is of major importance/requirement for the effective utilization of this substance. For this reason, a study of its emulsification properties was carried out, so as to obtain a clearer understanding of the mechanisms underlying the differences in the emulsification properties demonstrated by *A. senegal* and *A. seyal* gum exudates. The droplet size and mean specific surface area of gum Arabic oil-in-water emulsions prepared from the samples was determined by laser diffraction, using Mastersizer 2000 (Malvern Instruments), by measuring the angular variation in the intensity of scattered light as the laser beam passes through a dispersed particulate sample. The angular scattering intensity data was then analysed by the system to calculate the size of the particles responsible for the scattering pattern, using the Mie theory of light scattering as adopted by (Sochan, Polakowski and Lagod, 2014). The result of these experiments demonstrated that the Nigerian *Acacia senegal*-NG<sub>1</sub> gum sample droplet size, was smaller than that obtained for

the *Acacia seyal* sample. Furthermore, while the droplet size for the emulsion prepared using the *Acacia senegal*-NG<sub>1</sub> sample remained constant upon storage (for 1 week), the droplet size for the emulsion prepared using *Acacia seyal* increased. This result was as expected, since it is well known that *Acacia senegal* gums have superior emulsification properties compared to those harvested from *Acacia seyal* (Flindt et al., 2005). The fact that the *Acacia seyal* gum is able to form an emulsion at all, indicates that it is also able to adsorb at the oil-water interface, but to a lesser extent than gum harvested from *Acacia senegal*. Previous studies by Padala, Williams and Phillip, (2009) on the adsorption of *Acacia senegal* gum at the oil-water interface have reported that the adsorption is facilitated by the presence of proteinaceous components within the gum structure. The current study also demonstrated that *Acacia senegal*-NG<sub>1</sub> gum contained more protein than the *Acacia seyal* gum and consequently, is a better emulsifier than the *A. seyal* gum.

The hydrodynamic size of the molecules present in both the *A. senegal*-NG<sub>1</sub> and *A. seyal* gums in an aqueous state was also monitored using dynamic light scattering as a function of time, and it was found that molecular association/aggregation occurred. The extent of the association was shown to increase as the protein content of the gum sample increased and was inhibited in the



presence of an electrolyte (0.5 M NaNO<sub>3</sub>). It was therefore concluded that the association was due to electrostatic interaction between the protein moieties and glucuronic acid groups on individual macromolecules.

The adsorbed layer thickness of the gum Arabic adsorbed onto polystyrene latex particles was then determined by dynamic light scattering using the hydrodynamic principle. The hydrodynamic sizes of the polymer (gum Arabic) adsorbed was determined and the adsorbed layer thickness as a function of time was calculated from the difference in the hydrodynamic size of the particles with and without adsorbed polymer (gum). From the results, the thickness of the adsorbed layer was shown to increase as the amount of gum present increased. The adsorbed layer thickness also increased significantly with time. The most likely reason for this as discussed previously, is due to multilayer adsorption which occurs as a consequence of protein amine groups and the carbohydrate carboxylate groups within molecules present in solution interacting with similar oppositely charged groups within molecules adsorbed on the surface.

The adsorbed layer thickness for samples prepared with *Acacia seyal* in water and in the presence of 0.5 M NaNO<sub>3</sub> is much less than that of the *Acacia senegal*-NG<sub>1</sub> gum. As earlier observed, the *Acacia seyal* has a lower protein content than *Acacia senegal*, and its protein content is reported by Flindt et al., (2005) to be less

accessible as evidenced by the fact that it is not completely hydrolysed by proteolytic enzyme, hence the molecules will have a lower affinity for the hydrophobic surface, thereby making the molecules less attractive to each other.

The final section of the current study involved an investigation of the molecular composition of the gum Arabic samples in terms of their morphology/structure using transmission electron microscopy (TEM) and scanning transmission electron microscopy (STEM). The degree of aggregation of molecules present in the different gum samples over time was also investigated using these techniques. The results of this work showed that both the *A. senegal* and *A. seyal* gums comprised of an array of different sized molecules identified as putative AGP, AG and GP molecules (based on the literature). The sizes of the molecules observed ranged from ~12 nm for (GP), ~20 nm (AG) and ~60 nm for (AGPs). Immunogold negative staining (using JIM8 monoclonal antibody) indicated clear labelling of arabinogalactan proteins present in the gums harvested from *A. senegal*. However, the labelling of the *A. seyal* sample was inconclusive.

Both gum samples were also observed by TEM to form aggregates in aqueous solution over time which is consistent with the data presented in previous chapters. The aggregates of the *A. senegal* molecules formed more rapidly than those formed by the *A. seyal*

gum and had a snowflake-like appearance, whereas the *A. seyal* aggregates were more spheroidal in shape.

#### **5.2.0. Conclusion**

This study represents the first detailed comparison of Nigerian and Sudanese gum Arabic samples harvested from both *A. senegal* and *A. seyal*. The results of the study have shown that the *A. seyal* gum had a lower rhamnose and glucuronic acid content than the *A. senegal* samples, but had a higher arabinose content as has been reported previously (Anderson, *et al.*, 1990; Williams and Phillips, 2009). No significant differences were observed between the sugar compositions of the *A. senegal* gums from Sudan and Nigeria. However, the protein content of the Nigerian gums was shown to be significantly higher than observed for the sample originating from Sudan. In addition, all *A. senegal* gum samples were shown to contain at least twice the amount of protein as compared to the gum obtained from *A. seyal*, which in agreement with previous findings (Idris & Haddad, 2012).

The observed difference in protein content between the Nigerian and Sudanese *A. senegal* gums could be due to a variety of factors as discussed in Chapter 2. Most importantly, if this difference is found to be genotype specific (rather than due to other factors); then since the ability of *Acacia* gum exudates to stabilise oil-in-water emulsions is due to the proteinaceous components present

within the gum, then the Nigerian gum Arabic harvested from *A. senegal* trees of such a genotype may have enhanced emulsification properties (Padala, *et al.*, 2009).

In the current study, the *A. seyal* gum was shown to be a worse emulsifier than the *A. senegal* gum and was slower to aggregate. Both of which properties are most likely due to the lower protein content.

Therefore, considering the increased global demand for gum Arabic and the political instability in Sudan (World major supplier), the current study has provided strong evidence that gum Arabic sourced from Nigerian trees of *A. senegal* could provide a useful resource to meet this increased demand. In addition, with the current global glut of oil, and the resultant decrease in the price paid for crude oil, the Nigerian government has decided to focus more attention on agricultural aspects of its economy, in which an increase in good quality gum Arabic production could play an important role. Moreover, this study found that the Nigerian *A. senegal* trees produce gum of a higher protein content than those grown in Sudan, this may enhance the value of such gum for use in the food industry.

### **5.3.0. Further work**

The results of the current study could lead on to several lines of future research. A broader study of gum samples harvested from

*A. senegal* trees grown in commercial plantations in both Nigeria and Sudan would be of interest, in order to investigate whether or not the increased protein content of the Nigerian gum samples analysed in the current study is due to their genotype or environment. In order to study this in a more methodical manner, it would be best to harvest the seeds from such plants and grow, and harvest gum from them under controlled conditions, so that extraneous stress factors do not affect the results. Furthermore, relatively little is currently known with regards the process of gummosis and how the structure and molecular composition of the gum compares to the structure and composition of *Acacia* cell walls. A developmental study of the structure and composition of young *Acacia* seedlings through to gum producing maturity (five years of age) would also be of interest. Moreover, the TEM and STEM results presented in this study were preliminary, due to time constraints on this final aspect of the project. The results obtained indicate some intriguing differences in the aggregation properties of *A. senegal*-NG<sub>1</sub> and *A. seyal* gum molecules which require a more detailed study. In addition, a more extensive immunogold study, using a much larger panel of anti-plant cell wall antibodies would also be of interest.

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